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A Highly Sensitive Katharometer and its Application to the Measurement of Ethylene and Other Gases of Biological Importance

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Summary. The design and construction of a highly sensitive katharometer is described with special reference to the application of the instrument for the precise determination of traces of ethylene by gas chromatography. Factors influencing katharometer sensitivity, and the relative advantages and disadvantages of katharometers as compared with other types of detectors, are discussed.

Introduction

This communication describes the design and construction of a katharometer which has been found to possess more than 100 times the sensitivity of more conventional thermal conductivity instruments. By comparison with other types of gas analysers, the katharometer has the advantage of simplicity of design, and for this reason it is inexpensive to construct and relatively trouble free and long-lived in operation. The major disadvantage of available katharometers is that they can only detect quantities in excess of about 10^{-8} moles [4, 5, 12] and they are sensitive to temperature fluctuations. Recently instruments based on β-ray ionization [2, 8] and ionization at high voltage [4, 6, 11] have been described which are relatively independent of temperature and which are several orders of magnitude more sensitive than available katharometers (see Table I). Presumably these new detectors will largely supplant the katharometer for use in high-temperature gas chromatography. However, the katharometer may still be the most advantageous method for the detection of many biologically important substances which are volatile at room

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Table I. Sensitivities of various types of gas analysers

Instrument	Sensitivity (moles)	Author
Dielectric constant	10^{-7}	15
Katharometer	10^{-8}	4, 5, 12
Hydrogen flame	10^{-8}	13
Gas density balance	10^{-8}	9
β-ray ionization	10^{-12}	8
Other ionization	10^{-11}–10^{-13}	6, 10, 11

temperature. Included in this category are carbon dioxide, ethylene, various oxides of nitrogen, methane, ethane, oxygen, and other low molecular weight gases.

General Application

A sensitive katharometer operating with its block at room temperature can actually detect smaller amounts of most low molecular weight gases, such as those cited above, than can the β-ray ionization instrument, and it has almost the same sensitivity toward these gases as has been reported for most other types of ionization detectors. β-ray ionization can only achieve high sensitivities when the vapour to be analysed has an ionization potential below 11·6 eV, the excited state of argon.[8] The low molecular weight gases typically possess ionization potentials higher than that of argon, and the instrument shows only a small, negative response to them. Similarly, the sensitivity of other types of ionization detectors has been reported to be somewhat greater for heavier hydrocarbons than for lighter ones,[4, 6, 11] and it seems likely that this would also be true for other classes of compounds. For certain purposes, therefore, a highly sensitive katharometer has almost the same useful range as most of the other detectors now available, and this sensitivity is more than adequate for almost all biological problems which are encountered.

The katharometer has found wide application in the measurement of carbon dioxide.[1, 14, 16, 17] The instrument lends itself admirably to such determinations because when it is operated with air as a carrier gas it shows almost no sensitivity to oxygen.[3, 17] Consequently, chromatography is unnecessary and direct measurements of respired carbon dioxide can be made either in a continuously flowing air stream, in a confined system, or after short

collection periods. Both oxygen and carbon dioxide can be simultaneously measured if an 80 per cent helium–20 per cent oxygen mixture is used as a carrier gas. For such analyses the tissue is confined in a small chamber with the helium–oxygen mixture, and after a short collection period the chamber is flushed and the gases passed through a chromatography column inserted between the chamber and katharometer. If silica gel or carbon is used in the column, carbon dioxide is held back so that the first (negative) peak observed is due to oxygen consumption, and the second (positive) peak corresponds to carbon dioxide production (see Fig. 1).

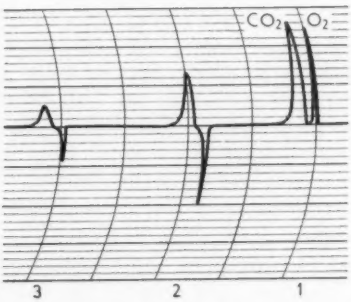

Fig. 1. Simultaneous determination of the oxygen consumption and carbon dioxide production of potato tissue. The graph reads from right to left and the distance between two vertical lines corresponds to 4 min. (1) A separation of oxygen and carbon dioxide on a 4 in. silica gel column using a 20 per cent oxygen–80 per cent helium mixture as carrier gas. (2) About 1 g of potato tissue has been confined in a tube with a 20 per cent oxygen–80 per cent helium mixture. The base line is obtained by by-passing the tube (using 3-way stopcocks) with the oxygen–helium mixture. After a 2·5 min collection, the contents of the tube were chromatographed; a negative peak for oxygen consumption and a positive peak for carbon dioxide production are observed. (3) Same as (2), except the tissue was confined for 1 min. Carbon dioxide readings were taken at 3 times the sensitivity of oxygen readings, and, on the basis of the areas under the curves, the R.Q. of the tissue is very nearly unity

In general, it has been our experience that by suitable modification the katharometer may be applied to almost any measurement involving a low molecular weight gas. In particular, we have used the instrument for the routine analysis of biologically produced ethylene, and for such a purpose it has proved to be about

10^4 times more sensitive than other methods which have been employed in the past.[18] Therefore, particular reference is made in this discussion to the application of the katharometer for the measurement of ethylene.

The Chromatography Column

Adsorption chromatography was found to be adequate at the low operational temperature (23°C) used for these analyses. Aluminium oxide was chosen in preference to other solid adsorbents such as silica gel and carbon because it produced sharper separations, minimized the spreading of gas at the rear of each band, and completely separated ethylene from carbon dioxide. 'Tailing' is caused by the non-linear adsorption isotherms of ethylene and many other gases, and to some extent by eddy diffusion and molecular diffusion on the column. Since the spreading becomes more intense with increasing retention times, it is advantageous to adjust the column length and the carrier flow rate to values which will yield the shortest retention time at which the particular substance is separated from all other components of the sample.

The gas flow circuit containing the chromatography column is shown in Fig. 2. The flow of the carrier gas, helium, is controlled

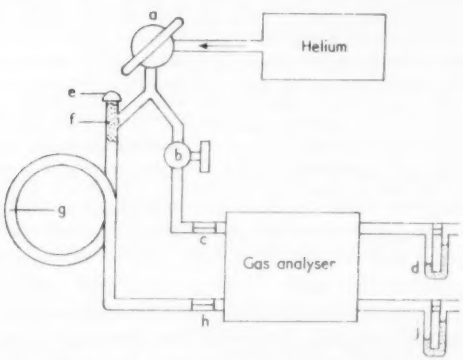

Fig. 2. Gas flow circuit; (a) reduction valve, (b) needle valve, (c) capillary, (d) flow meter, (e) vaccine cap, (f) cotton, (g) chromatography column, (h) capillary, (j) flow meter

by a reduction valve (a), after which the line splits into control and sample sides. The latter is fitted with a vaccine cap (e) through which the sample is inserted by means of a hypodermic syringe. A cotton plug (f) prevents any extraneous liquids from reaching the surface of the adsorbent column (g). The chromatography column is an 18 in. piece of $\frac{1}{4}$ in. bore Tygon tubing packed with aluminium oxide. A small piece of cheese cloth stretched over the end of the column is held in place by inserting a capillary tube (h). The cloth prevents the escape of aluminium oxide, and the capillary helps to reduce flow changes. The sample line passes through the gas analyser and terminates in a flow meter (j). The control side contains a needle valve (b) which is adjusted to balance the flow resistance of the chromatography column; from this the helium passes through a capillary (c) to the gas analyser, and emerges from the flow meter (d). The flow rate was normally maintained at 27 c.c/min, although slightly higher or lower flow rates in no way affected the performance or sensitivity of the apparatus.

Construction of the Katharometer

Fig. 3 contains a schematic wiring plan of the katharometer. The 108 ohm resistors shown in the diagram represent the tungsten filaments used as detecting elements. A second bridge, consisting of two fixed 100 ohm resistors and two variable resistors, was wired into the side of the filament bridge as a balancing device. The 5 ohm potentiometer was used for coarse adjustment, and the 400 ohm potentiometer for fine adjustment. The filaments were heated by current from storage batteries; these should be of good quality because the limit of sensitivity of the apparatus may be determined by battery drift. A 500 ohm potentiometer was used to adjust the battery current. The signal from one corner of the filament bridge passes to a five-way switch where fixed resistors (0, 250, 1k, 10k, and 1M ohm) can be selected according to the sensitivity desired. A Perkin–Elmer d.c. breaker amplifier was used to amplify the katharometer output; a 5 ohm manganin shunt was wired across the amplifier input circuit to reduce the input impedance. The amplified signal was filtered and fed into an Esterline–Angus 0–0·1 mA d.c. recording galvanometer.

When a 0·2 megohm resistance (see Fig. 3) is shunted across one arm of the filament bridge, a test signal is produced which can be used to calibrate the sensitivity selector. The sensitivity can also be controlled by the gain of the amplifier.

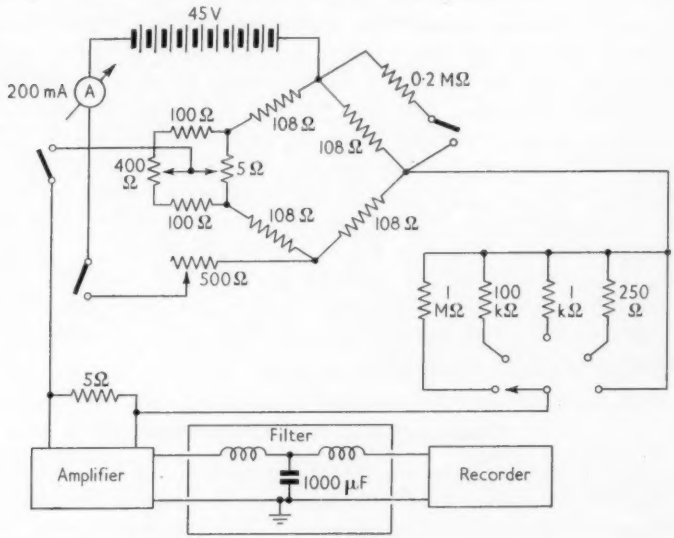

Fig. 3. Schematic wiring diagram of katharometer

The construction of the thermal conductivity cell is shown in Fig. 4. Four holes, the filament channels, were bored length-wise through a cylindrical aluminium block. At one end of the block (section AB) the longitudinal channels were intersected by four holes tapped from the outside; these were threaded and nozzles screwed into place. At the opposite end (section EF), a hole was drilled from the outside through the entire block to intersect two channels. This was threaded on both ends and brass screws were inserted until their ends abutted on the central passages. These screws sealed the block so that the two channels were internally connected. A second hole, at a right angle to the first but closer to the end of the block (section GH), was made to connect the remaining two channels internally. When the ends of the

longitudinal passages are sealed, gas entering one nozzle passes the length of the block, crosses over to the channel on the other side, flows back the length of the block, and emerges on the opposite side from which it entered. Two such routes exist; one corresponds to the control side and one to the sample.

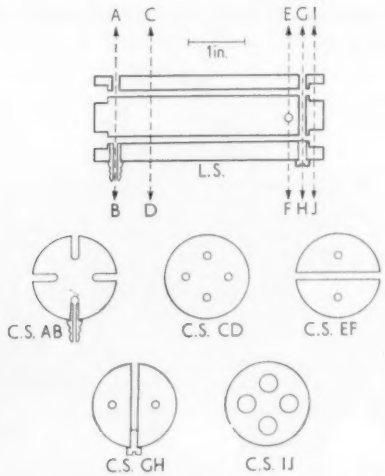

Fig. 4. Construction of katharometer block

Filaments from 20 W light bulbs were used as the 'hot wires' of the katharometer. Since test lighting causes tungsten to crystallize and become brittle, it is best to obtain the filaments before they have been inserted in light bulbs. The connections between the filaments and supporting leads were found to be critical—in fact the noise level of the entire instrument can be determined by fluctuations in the resistances of these joints. The coiled filaments are too fine to be directly spot-welded, but a satisfactory joint was made by means of a cold press followed by a light spot-weld. One end of a 3 in. piece of 17 gauge nickel wire (see Fig. 5 (a)) was filed to a flat surface and grooved to a depth of $\frac{1}{8}$ in. with a diamond saw. A filament (see Fig. 5 (c)) was inserted into the groove, carefully aligned, and held in place by a cold press $\frac{1}{16}$ in. from the end of the nickel lead. A spot-weld applied over

the cold press (see Fig. 5 (b)) caused the nickel to flow into a few coils of the filament. A second lead was joined to the opposite end of the filament in a similar manner.

Fig. 5 illustrates the suspension of the tungsten filaments. Eight aluminium plugs (d) were threaded to fit the larger holes tapped in the ends of the block where the channels terminate. The centres of these plugs were drilled out, and holes bored from the outside to the centre were threaded to hold set screws (j).

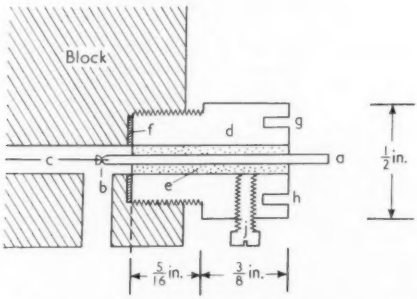

Fig. 5. Suspension of filaments in katharometer block

Teflon sleeves (e) were fitted into the mounting plugs, and holes just large enough to admit nickel leads were drilled longitudinally through the insulation. Tygon washers (f) approximately $\frac{1}{32}$ in. thick were inserted in the mounting holes where they formed an air seal when the plugs were tightened. Two shallow holes (g, h) were drilled in the ends of the mounting plugs, and a special wrench with two prongs appropriately spaced was used for tightening.

For mounting the filaments, it was found convenient to support the aluminium block vertically in a vice. One lead was slipped into a mounting plug and the free hanging end was lowered into its channel. The plug was inserted, the lead drawn up until the filament was centred, and the set screw tightened against the Teflon to secure the lead. The second mounting plug was slipped over the lower lead and screwed into place; before the set screw was inserted, the lead was drawn out slightly to stretch the filament. At this time each filament was tested with an ohmmeter

to be certain that it was not short-circuited to the block. After the block was completely assembled, a drop of collodion was applied over each screw head and joint to make it air tight.

To prevent draughts from reaching the block it was mounted inside a metal box (see Fig. 6). The battery leads and amplifier connections were made of shielded coaxial cable and entered the instrument through phonograph jacks.

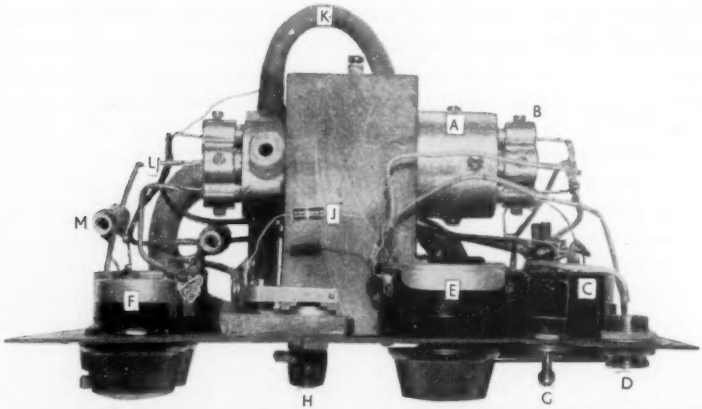

Fig. 6. The casing of the katharometer has been removed to reveal the aluminium block and the details of wiring; (A) aluminium block, (B) mounting plug, (C) ammeter, (D) battery input, (E) 500 ohm potentiometer for regulating filament current, (F) 5 ohm balancing potentiometer, (G) battery switch, (H) test switch, (J) 0·2 megohm test resistor, (K) rubber tubing (attaches to nozzle on casing), (L) nickel filament lead, (M) 100 ohm fixed resistor of the balancing bridge

Conditions for Maximum Sensitivity

When a gas having a small trace of impurity is compared with a pure gas which forms the major constituent, ignoring heat losses due to radiation, convection, end-cooling, gas flow, etc., and assuming that the temperature coefficient of thermal conductivity is the same for the mixture as for the pure gas, to a first approximation the sensitivity of a katharometer can be expressed by the equation:[3, 7]

$$E = \frac{I^3 R_0 R_t \alpha \epsilon}{16 J k K_0} \frac{2\gamma}{(1+\gamma)} \qquad \ldots\ (1)$$

where E is voltage unbalance at the corners of an open circuited bridge, I is current in the circuit, R_0 is resistance of the filament at O°C, R_t is resistance of the filament at t°C, α is temperature coefficient of resistivity of the filament, γ is ratio between resistances of opposite arms of the bridge, J is mechanical equivalent of heat, K_0 is thermal conductivity of pure gas at O°C, k is rate of heat loss of filament per unit temperature difference between the wire and the channel in which it is suspended, in gas of unit conductivity, and ϵ is relative change in thermal conductivity due to the addition of the impurity to the pure gas.

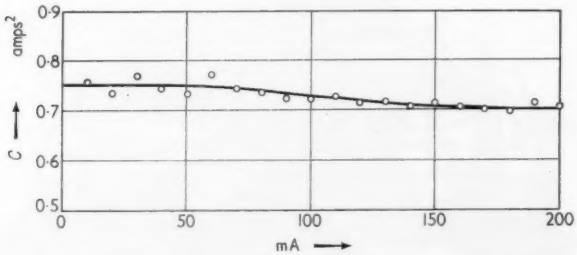

Fig. 7. Sensitivity as a function of filament current. Data were obtained using a Kin–Tel model 204A electronic galvanometer (made by Kin–Tel Co. San Diego, California) in place of the Perkin–Elmer amplifier shown in the wiring diagram (Fig. 3). Since the Kin–Tel amplifier is relatively independent of input impedance, the 5 ohm input shunt could be removed to allow the katharometer bridge to develop its full output voltage. R_t has been calculated from measured values of I and the voltage drop across the filament bridge. V corresponds to the peak voltage produced with a 500 μl. sample of air

If k and ϵ are assumed to be constant (they actually have a temperature coefficient), then for a particular instrument:

$$C = \frac{I^3 R_t}{V} \qquad \ldots\ (2)$$

where V is observed bridge output (V), and C is a constant (amps²).

Fig. 7 contains calculated values of C as a function of current and the peak voltage output obtained with a 500 μl. sample of air. Equation 2 is based on the assumption that the temperature coefficient of thermal conductivity of the pure gas is the same

as that of the mixture, but it is known that thermal conductivities converge at higher temperatures.[3] The observed slight decrease in the value of C at higher currents is probably due to this convergence. The effect is still very small even at 200 mA where the wire temperature is 150 °C, so for all practical purposes it can be disregarded throughout the operational temperature range. However, as Keulmans has pointed out,[7] the stability of the katharometer decreases and the noise level increases as the current is raised, so an optimal wire temperature exists above which any increase in temperature will result in an actual decrease in usable sensitivity. The noise level of the instrument is difficult to determine at low filament currents, but between 135 and 150 mA the noise level increased at about the same rate as the sensitivity, while between 150 and 180 mA it increased slightly more rapidly than the signal. In practice, however, katharometer drift may be considerably more important than noise, and this seems to be particularly true of tungsten wire katharometers.[7] One cause of drift is water vapour in the carrier gas, and performance is improved by drying this gas. Another cause of drift is battery discharge, and it might be expected that the signal to battery-drift ratio would be improved by raising the current. In the present case the signal/drift ratio was found to improve up to a current of 180 mA, but above this value the drift worsened so rapidly as to make higher currents unfeasible. An operating current of 170 mA was found to be about optimal when all three factors, signal, drift, and noise, were taken into account. In order to achieve this condition it is essential that the noise level of the amplifier and recorder circuit be lower than the noise level of the katharometer at the filament current which yields maximum sensitivity.

Although careful selection of the operating conditions can maximize the sensitivity of a katharometer, an even more important consideration is the design of the instrument. The voltage output per unit change in thermal conductivity can be increased by reducing the filament radius and channel radius, and by selecting filaments which are as long as possible and which possess a high temperature coefficient of resistivity.[3, 7] The present instrument employed the longest coiled filaments which were feasible from a constructional point of view. They were

made of 10 μ tungsten wire, this being the smallest radius to which tungsten can be drawn without excessive loss of tensile strength. Tungsten is a particularly suitable material because it can be drawn to about half the width of platinum and it possesses a slightly higher temperature coefficient of resistivity than platinum. The 3 mm diameter channels used in the block were the smallest bore in which the coiled filaments could be supported without danger of short circuiting against the walls of the block.

Results

The most widely accepted method of expressing katharometer sensitivity is in terms of the following equation:[4, 5]

$$S = \frac{A \times C_1 \times C_2 \times C_3}{W} \quad \ldots . \quad (3)$$

where S is the sensitivity parameter (ml × mV per mg), A is peak area (cm^2), C_1 is mV per cm of chart, C_2 is chart speed (min/cm), C_3 is flow rate (ml/min), and W is weight of sample (mg).

Since the voltage output obtained for a particular weight of sample can be increased by raising the current, any expression of sensitivity must also include a statement of the noise level of the instrument under the particular operating conditions. Hot wire katharometers generally have sensitivities falling in the range 300–1,000 mV × ml per mg with noise levels ranging from 5–20 μV,[4, 5] and thermistor katharometers vary from 100–600 mV × ml per mg with noise levels in the vicinity of 10 μV.[4] The instrument described here possesses a sensitivity of 35,000 mV × ml per mg with a noise level of 3 μV when it is operated at 170 mA current. This represents a gain in sensitivity over other katharometers of about 100 times. The analyser has been used for detecting quantities of nitrous oxide or of ethylene as small as 10^{-10} moles (see Fig. 8), and at this sensitivity the base line is fairly stable. One difficulty is the trace of air which is still leaving the sample chamber at the time ethylene enters. This creates a slope in the base line just in front of the ethylene peak, but the exact extent of air contamination at the time of the reading can be estimated by drawing a smooth curve connecting the slope with the final base line. The amount of ethylene can then be calculated by deter-

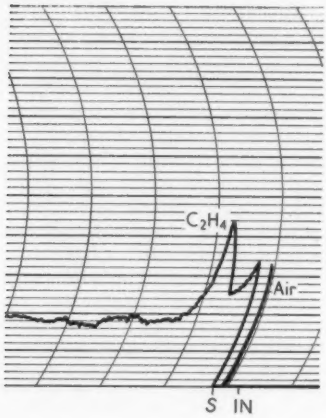

Fig. 8. A fractionation of 10^{-9} moles of ethylene from 0·25 ml of air. The sensitivity has been increased 10^4 times at the point marked *S*. Full scale corresponds to 140 μV and the distance between two vertical lines equals 4 min. The flow rate was 28 ml of helium per min. $S = 35{,}000$ ml × mV per mg with a noise level of *ca.* 3 μV. Record was obtained at 170 mA filament current using a Kin–Tel model 204A electronic galvanometer for amplification

mining the value of the theoretical base line at the time the ethylene was causing its maximum reading, and subtracting this value from the peak height. This difficulty could also be alleviated by using a longer chromatography column to improve the separation of ethylene from air. However, peak height is the factor which ultimately determines the minimum amount of gas which can be detected, and although areas are fairly constant regardless of column length and carrier gas flow rate, peak height decreases with an increased retention time. Actually a factor of about 2 in usable sensitivity can be gained by minimizing the retention time. For both air and ethylene the instrument showed a linear relationship between peak height and quantity, from the smallest standards which could be prepared accurately (0·1 μl) up to 1 ml of gas. Measurements are reproducible to within 1 per cent error. For example, three successive chromatograms of 0·5 ml samples of air which were withdrawn from an apple with a syringe gave values for the percentage of ethylene in the

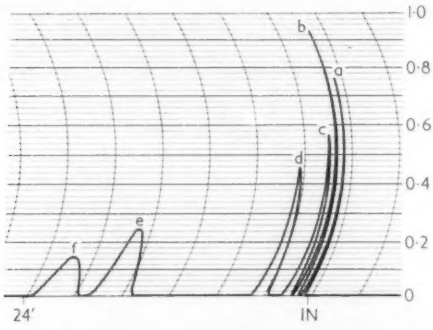

Fig. 9. A fractionation of 1·5 ml of natural gas to which 0·25 c.c. of ethylene has been added. The peaks are: (a) methane, oxygen, nitrogen and carbon dioxide; (b) ethane; (c) ethylene; (d) propane; (e) *iso*butane; and (f) *n*-butane. The sensitivity has been increased 33·2× between (a)–(b), reduced 7·3× between (b)–(c), increased 535× between (c)–(d), and increased 4,000× between (d)–(e). (By courtesy of *Proc. Nat. Acad. Sci.*, 45 (1959), 336)

internal atmosphere of 0·163, 0·160, and 0·163 respectively. Fig. 9 illustrates a fractionation of several hydrocarbons and demonstrates the stability of the analyser over a wide range of sensitivity changes.

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Development of *Streptomyces aureofaciens* in Submerged Culture

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Summary. During the biosynthetic production of chlortetracycline on a medium with sucrose, soy meal, corn steep extract, sodium chloride, ammonium sulphate, calcium carbonate and beet molasses, the amount of nucleic acids synthesized by the culture and the maximum respiration of the culture is determined by the amount of inorganic phosphate in the original medium. Chlortetracycline production does not start until all the inorganic phosphate has been consumed. The adverse influence of increased amounts of inorganic phosphate upon production of chlortetracycline is more apparent in flasks on a shaking machine than in agitated and aerated fermentation tanks with higher oxygen transfer numbers.

Introduction

The kinetics of microbiological growth and development have so far been studied only for the bacteria. For these they have been worked out in such detail that the various relationships can be expressed in exact mathematical form.[1, 2] Filamentous microorganisms, however, are much more difficult to study. In this communication we wish to present our findings concerning some factors determining the development of submerged cultures of *Streptomyces aureofaciens* and the production of the antibiotic chlortetracycline. Special attention was paid to the influence of phosphate, because it has been stated previously[3] that increased concentration of phosphate inhibits the biosynthesis of chlortetracycline.

In laboratory experiments the strain *Streptomyces aureofaciens Bg*, producing approximately 1,000 μg chlortetracycline/ml, was used while strain *S. aureofaciens BMK*, producing 2,000–2,400 μg/ml, was employed in tanks. Spore and vegetative inocula were prepared according to Herold *et al.*[4] and the medium

contained: sucrose 2 per cent, corn steep solids 0·24 per cent, soy-bean meal 2 per cent, ammonium sulphate 0·5 per cent, calcium carbonate 0·4 per cent, sodium chloride 0·5 per cent, beet molasses 0·2 per cent.

The soy-bean meal used was the product of an extraction process and contained about 1 per cent oil. In the laboratory experiments soy-bean meal was substituted by a soy-bean extract so that the dry weight of the mycelium could be determined during cultivation. This extract was prepared by boiling 30 g of soy-bean meal in 750 ml of water for 10 min. The extract was then filtered off and sterilized separately. After sterilization (120°C, 30 min) a sterilized solution of the other raw materials was added to the flasks so that this medium composition closely resembled that of the suspension medium used in the tanks.

Laboratory experiments were performed on a laboratory shaker (96 c/min, amplitude 10·5 cm), in 500 ml flat bottom flasks and pilot-plant experiments were performed in tanks containing 200 l. of medium, equipped with a ring sparger and a turbine impeller turning at 200 rev/min. In all experiments the rate of oxygen absorption was determined by the sulphite oxidation method.[5] In flasks it amounted to 520 ml O_2/l.h; in tanks 1,200 ml O_2/l.h. The tanks were aerated with 100 l. of air/min. Cultivation temperature in all experiments was 29°C.

During cultivation, changes in medium composition, dry weight of mycelium, respiration and synthesis of nucleic acids were followed.

Production of chlortetracycline was determined colorimetrically[6] or biologically by the plate diffusion method using *B. subtilis 6633* as a test organism.[7] Sucrose was determined after acid hydrolysis with 2 per cent HCl (10 min at 100°C) by the method of Bertrand. Alpha-amino nitrogen was determined by the method of Schroeder *et al.*,[8] ammonia nitrogen according to Cole and Parks[9] and total nitrogen after kjeldahl digestion by the same method. Pyruvic acid was determined according to Friedemann and Haugen.[10] Nucleic acids were extracted from the mycelium by perchloric acid according to Ogur and Rosen[11] and their concentration determined spectrophotometrically[12, 13] on the Universalspektrophotometer Zeiss. Respiration of the mycelium was measured either directly by the Warburg method or polarographically[13] (the respiration values shown in Figs. 1, 3

and 4 were obtained by the polarographic method). Phosphorus was determined according to Fiske and SubbaRow.[14] Whenever this method gave turbid solutions, the extraction method of Ging[15] was substituted.

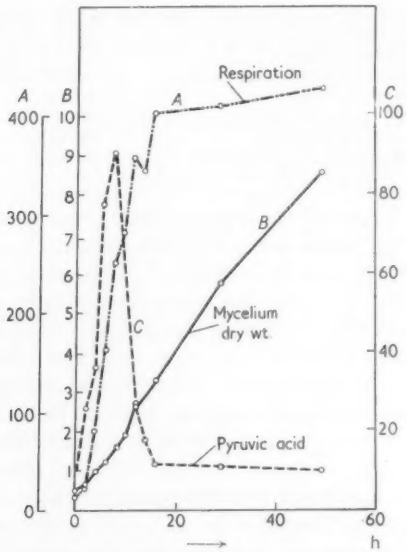

Fig. 1. Growth, respiration and pyruvic acid level during the cultivation of *Streptomyces aureofaciens* in shaken flasks. *A*, respiration (ml O_2/l. culture/h); *B*, dry wt. mycelium (mg/ml); *C*, pyruvic acid (μg/ml)

Experiments and Results

Composition of the medium determined by analysis is given in Table I; for all practical purposes it contains no reducing sugars. Quantitatively, the most important source of nitrogen is the ammonia and protein nitrogen of soy-bean meal, whereas free amino acids from corn steep solids are less important. The medium contains large amounts of organic phosphorus, mainly phytin from corn steep liquor, which is relatively insoluble at pH 6·8–6·9. Inorganic phosphorus also originates mainly from the corn steep solids and is mostly present in a soluble form.

Table I. Composition of the nutrient medium used in 200 l. fermentors

Reducing sugars after hydrolysis	30–32 mg/ml
Free reducing sugars	0·0 mg/ml
Lactic acid	1·1–1·2 mg/ml
Pyruvic acid	10–15 μg/ml
Total nitrogen	2·2–2·6 mg/ml
Ammonia nitrogen	0·9–1·1 mg/ml
a-Amino nitrogen	0·23–0·25 mg/ml
Total phosphorus	200 μg/ml
Soluble phosphorus	64 μg/ml
Inorganic phosphorus	34–41 μg/ml
Soluble inorganic phosphorus	30–37 μg/ml

The initial phase of the fermentation, lasting about 10 h, is characterized by rapid and complete consumption of inorganic phosphate (Fig. 3), and by increase of the level of pyruvic acid in the medium (Fig. 1). Sucrose, ammonia nitrogen and lactic acid are used up only slightly during this period. Rapid consumption of these nutrients does not take place until the following phase of fermentation. In laboratory experiments (shaking machine) sucrose was not consumed until the 96th h; in tank experiments consumption was complete in 48–60 h. Ammonia nitrogen was consumed in approximately the same time. The pyruvic acid level reached a maximum (90–120 μg/ml) at about the tenth hour, decreasing rapidly thereafter to 20 μg/ml and remaining at this level without appreciable change until the end of the cultivation (Fig. 1).

In laboratory flask experiments with a 'non-suspension' medium, mycelium weight increased uniformly from the start for about 50 h, when growth ceased. At this time the dry weight of mycelium had reached a value of 8–12 mg/ml. Respiration of the culture (expressed in ml O_2 consumed by 1 l. of medium in 1 h) rose exponentially during the first 10 h of fermentation, reaching a value of 250–350 ml O_2/l.h. After this, respiration increased no further; on the contrary, after the 48th h a slight decrease in respiration was observed. During the tenth hour, with respiration at a maximum, mycelial weight was still very small—only about 2 mg/ml. The specific respiration rate was therefore at a distinct maximum at this time (Q_{O_2} = 100–150 ml O_2/g dry wt. mycelium/h). Since the dry weight of mycelium

subsequently increased about fivefold, with total respiration remaining constant, the value of the specific respiration rate (Q_{O_2}) decreased to 20–40 ml O_2/g dry wt. mycelium/h.

Nucleic acids were synthesized during the first 10 h of fermentation; in the subsequent phases their amount remained approximately constant. RNA reached a maximum after 10 h of cultivation, after which it decreased, whereas the DNA continued to increase slightly (Fig. 2). Growth of mycelium after 10 h was

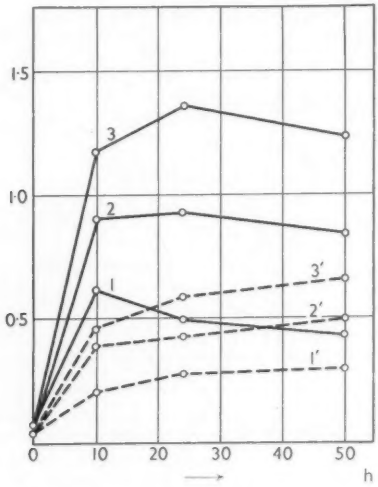

Fig. 2. Nucleic acid contents (mg/ml) in whole broth during the cultivation of *Streptomyces aureofaciens* in shaken flasks for various levels of inorganic phosphorus in the nutrient medium prior to fermentation: 1 and 1′, 30 μg P_i/ml (standard medium); 2 and 2′, 60 μg P_i/ml; 3 and 3′, 90 μg P_i/ml. Full lines: RNA; broken lines: DNA

not accompanied by parallel accumulation of nucleic acids, but only by a partial transformation of RNA to DNA. The nucleic acid content in unit weight of dry mycelium decreased considerably, especially for RNA.

The first traces of chlortetracycline were not detected until all of the inorganic phosphate was used up, pyruvic acid reached its maximum level and respiration of the culture ceased to increase.

Synthesis of chlortetracycline then proceeded in a roughly uniform manner until sucrose and ammonia nitrogen were depleted. (Figs. 3 and 4).

During cultivation on media containing two or three times the usual concentration of inorganic phosphate, added in the form of

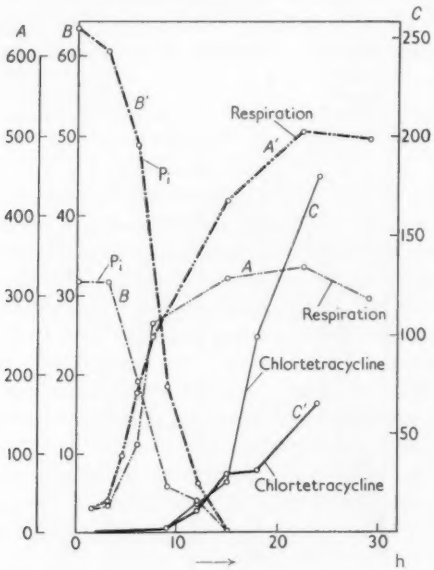

Fig. 3. Respiration, phosphate level and chlortetracycline production during the early hours of cultivation of *Streptomyces aureofaciens* in shaken flasks. *A*, respiration (ml O_2/l. culture/h); *B*, level of inorganic soluble phosphorus (μg P_i/ml); *C*, chlortetracycline (μg/ml). *A*, *B*, *C*, standard nutrient medium with 30 μg P_i/ml; *A'*, *B'*, *C'*, standard medium enriched with inorganic phosphate to the concentration of 60 μg P_i/ml

KH_2PO_4 (i.e. 60 or 90 μg P/ml as compared with the usual 30 μg/ml), it was found that the mycelium completely consumes even this increased amount of phosphate in the same time as the normal 30 μg/ml. The velocity of consumption of inorganic phosphate is thus proportional to its concentration. The amount of nucleic acids synthesized by the mycelium is, in turn, proportional to the concentration of inorganic phosphate in the

medium and the course of their synthesis does not differ from that in the basic medium with a normal amount of phosphate. Here, too, RNA reaches its maximum in the early hours of cultivation, after which it decreases slightly, whereas DNA continues to increase slowly (Fig. 2). The cultures on media with increased phosphate level showed a considerably increased consumption of

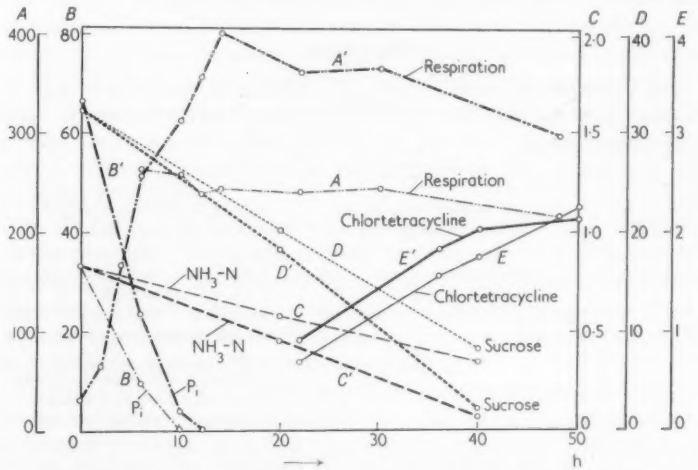

Fig. 4. Metabolism of a culture of *Streptomyces aureofaciens* in a 200 l. fermentor. *A*, respiration (ml O_2/l. culture/h); *B*, level of inorganic soluble phosphorus (μg P_i/ml); *C*, ammonia nitrogen (mg N/ml); *D*, reducing sugars after hydrolysis (mg sucrose/ml). *E*, chlortetracycline (mg/ml). *A*, *B*, *C*, *D*, *E*, standard nutrient medium; *A'*, *B'*, *C'*, *D'*, *E'*, standard medium enriched with inorganic phosphate to the total concentration of 60 μg P_i/ml.

oxygen compared with cultures on the normal medium. On a medium containing 30 μg of inorganic P/ml, oxygen consumption amounted to 250–350 ml O_2/l.h, while on a medium with 60 μg of inorganic P/ml it reached a value of 500–600 ml O_2/l.h—almost twice as high (Figs. 3 and 4).

The same dependence of the maximum consumption of oxygen upon the amount of inorganic phosphate in the original medium was observed in flasks and in 200 l. fermentation tanks. Also the absolute values of oxygen consumption were approximately equal in both series of experiments. A substantial difference was

observed, however, in the production of chlortetracycline. In shaken flasks, doubling of the original phosphate level (to 60 μg/ml) decreased production of chlortetracycline to 40 per cent of control (on the average). On the other hand no such decrease was observed in fermentation tanks; in fact, in fermentation tanks with media enriched with phosphate, maximum production was reached somewhat earlier than on the normal medium (Fig. 4).

Discussion

On the basis of these results the cultivation cycle of *S. aureofaciens* may be divided into two phases, characterized by the following features:

Cultivation phase	Changes in the composition of medium	Changes of the properties of the mycelium
0–10 h	(a) total consumption of inorganic phosphate (b) increased level of pyruvic acid	(a) growth of mycelium up to 2 mg/ml dry weight (b) synthesis of nucleic acids, reaching the maximum RNA level (c) exponential increase in respiration; maximum Q_{O_2} value (d) no chlortetracycline production
10–50 h	(a) consumption of sucrose and ammonia nitrogen (b) rapid consumption of pyruvic acid (c) zero level of inorganic phosphate	(a) growth of mycelium up to 8–12 mg/ml dry weight (b) no change of total amount of nucleic acids; decrease in RNA with DNA increasing slightly (c) no change in oxygen consumption, later decreasing slightly; Q_{O_2} drops sharply (d) biosynthesis of chlortetracycline until consumption of sucrose and ammonia nitrogen is complete

The characteristic course of nucleic acid synthesis and the maximum in the RNA level observed here were previously described by Guberniev *et al.*[16] Our experiments indicate that these changes are connected with the level of inorganic phosphate in the medium. The presence of soluble inorganic phosphorus is a necessary condition for the synthesis of nucleic acids and for

increased respiration, but not for the growth of mycelium. On the contrary, mycelial weight increases fivefold in the period between 10 and 50 h without any increase of the level of nucleic acids or increase in oxygen consumption. The decrease in specific respiration rate of *Streptomyces aureofaciens* has already been observed by Boretti and Raggi[17] and is believed to result from the inhibition of respiration by the chlortetracycline produced in the culture. It must be noted, however, that the decrease in Q_{O_2} is the result of an increase in mycelial dry weight, not of a decrease in total oxygen consumption by the culture. It appears, then, that the decrease in Q_{O_2} after depletion of inorganic phosphate results from the fact that growth of the new mycelium is not, under these circumstances, accompanied by synthesis of respiratory enzymes.

A similar correlation between phosporus uptake and the synthesis of nucleic acids and respiratory enzymes was observed in the cultures of *S. rimosus* on a medium with glucose, starch and corn steep liquor. In this case, however, the development of the culture was a little more complicated because of the presence of two different carbon sources in the medium.[13]

Cultures grown on a medium with increased inorganic phosphate level show increased respiration, and their total metabolism expressed in terms of sucrose and ammonia nitrogen consumption is considerably accelerated. Acceleration of metabolism by the addition of inorganic phosphate has already been observed by Di Marco *et al.*[18] From our experiments it follows that increased respiration and acceleration of total metabolism is observed at a time when inorganic phosphate has already been totally consumed. The accelerating effect of phosphate cannot, therefore, be explained by the direct influence of the phosphate ion upon the velocity of enzymatic reactions; inorganic phosphate probably acts as a factor limiting the synthesis of nucleic acids and also the synthesis of some respiratory enzymes.

Early consumption of inorganic phosphate is a necessary condition of biosynthesis of chlortetracycline. Mycelium developing in the presence of inorganic phosphate and synthesizing nucleic acids and respiratory enzymes does not produce chlortetracycline. It is not until synthesis of nucleic acids stops, as a consequence of depletion of inorganic phosphate, and respiration

of the culture ceases to increase, that synthesis of chlortetracycline starts.

The differences observed between the effects of increased inorganic phosphate levels upon the production of chlortetracycline in flasks and in fermentation tanks can be explained by the difference between the oxygen transfer in these two types of fermentation vessels. In flasks on a shaking machine under our experimental conditions, the rate of dissolution of oxygen was 520 ml O_2/l.h. For cultures with a normal amount of phosphate (30 μg/ml) reaching a maximum respiration of 250–350 ml O_2/l.h the supply of oxygen is sufficient. By increasing the level of phosphate to 60 μg/ml respiration of the culture increases to 500–600 ml O_2/l.h, and in this case the supply of oxygen in the flask on the shaking machine is no longer sufficient and production of chlortetracycline is strongly suppressed. In a 200 l. fermentation tank, under the experimental conditions described, the rate of dissolution of oxygen amounts to 1,200 ml O_2/l.h. In this case the supply of oxygen is sufficient even for the culture grown on a medium with increased level of inorganic phosphate, and consequently chlortetracycline production remains unaffected.

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Separation of Acid Polyelectrolytes and Proteins in Cellular Extracts—I*

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Summary. An electrophoretic method for the separation of acid polyelectrolytes from proteins in cell extracts is described.

A description of the apparatus is given and some results of the separation of extracts from *Bordetella pertussis* are included.

Introduction

The main purpose of this paper is to describe a preliminary fractionation suitable for handling total extracts from cells, and for resolving the macromolecular components to a stage appropriate for storage and from which further purification may be undertaken.

Bordetella pertussis was chosen as test organism since the work was planned in conjunction with a team of microbiologists; the aim was to test different macromolecular fractions from immunological and toxicological points of view.

Considerable success has recently been achieved in the separation of proteins; three main methods having been used. E. Cohn's classical methods for the separation of proteins are based on the different solubilities of serum proteins at different temperatures, hydrogen ion concentrations, ionic strengths of buffers and organic solvents. Tiselius's electrophoresis methods have been shown to be of great value. A modification of this technique has been described by Porath,[1–3] employing electrophoresis on stabilized media in columns. Sober and Peterson[4–5] have developed methods for the separation of proteins on cellulose ion

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exchangers, using hydrogen ion concentration gradients as well as other ionic strength gradients.

These methods have been developed mainly on the nucleic acid-free material serum, but as soon as the material to be separated contains nucleic acids or acid polysaccharides separation of all proteins is difficult. In cell extracts part of the proteins will easily be precipitated with nucleic acid material. Our aim has been to find a way to remove nucleic acids and other acid polyelectrolytes before separation of the proteins was attempted. For this purpose we have used the electrophoresis technique developed by Porath,[1-3] but in order to separate proteins from nucleic acids the column must be at least 3 m long. Some modification of Porath's technique then had to be made because of the length of the column. In order to reduce enzymic reactions during and after disruption of the cells we have worked at low temperatures and have tried to cut down the time for preparations. Merthiolate was present in a concentration of 1/10,000 (w/v) in all fractions, except during electrophoresis.

The main preparative methods we have used are: (1) Mechanical disruption of the cells; (2) Ultra-filtration and/or freeze-drying; (3) Electrophoresis on columns stabilized with ethanolized cellulose; and (4) Chromatography (mainly on diethylaminoethyl cellulose).

Preparation of Biological Material

The cells were separated from cultures by centrifugation at $4{,}000 \times g$ for 90 min at 15°C. They were usually freeze-dried and were disintegrated at −15°C on a magnetovibration table (50 c/s, amplitude 1 mm) for 36 h in 30 per cent pre-cooled ethanol (v/v) in plastic stoppered, heavy Pyrex glass tubes (inner dimensions 30×100 mm) filled with glass beads (5 mm diam). The amount of lyophilized material introduced into each tube was 2 g. The suspension was lyophilized after disintegration, extracted with water or salt solution and centrifuged for 15 min at $100{,}000 \times g$; the extraction being repeated three times. The combined extracts were freeze-dried and stored over a desiccant *in vacuo* at +4°C. In recent preparations freeze-drying has been omitted, disruption by vibration then taking 140 h. It is planned to use a Hughes press for disruption.[6] All extracts are at present being

concentrated by ultra-filtration, frozen down and stored at −20 °C. Estimated on a dry-weight basis, between 20 and 25 per cent of the cell material was extracted in this way. Freeze-drying, freezing and thawing, and dialysis against low ionic strength salt solutions all brought about denaturation of at least some of the proteins, and for this reason extensive use of ultra-filtration was introduced. Cell extracts may thus be concentrated to a thick paste and then diluted to a clear solution. When the extract was freed from most of the water and dialysable substances by ultra-filtration the hydrogen ion concentration and ionic strength were adjusted to the values which were to be maintained during the subsequent electrophoresis.

Methods

Ultra-filtration

Dialysis tubing was fitted as tightly as possible over a rubber stopper. The tubing was filled with water and Pyrex beads (0·3 mm diam.) were then poured into it. After the beads had settled and were tightly packed, Pyrex glasswool was pressed down on the beads and the open end of the tubing was fitted over a rubber stopper equipped with a short glass tube. That part of the dialysis membrane surrounding the rubber stopper was protected by masking tape and stainless steel wire wound tightly around it. When suction was applied to the glass tube and the whole apparatus (length 200 mm, width 20 mm) dipped into a cell extract, 9–10 ml of solution was filtered per hour (24 suction bodies filtered about 2 l. in 24 h). These suction bodies can conveniently be fitted in different vessels to suit various volumes of extract, electrophoresis, and chromatography fractions, and bodies made with 6·35 mm wide dialysis tubing are suitable for the last stage concentration. The ultra-filtration method for volume reduction seems to give little denaturation of proteins as judged by the insignificant amount of protein precipitating out of the solution.

Construction and Handling of Electrophoresis Apparatus

The apparatus (Figs. 1–7) was constructed according to Porath[1–3] with modifications necessitated mainly by the length and the difficulties of packing a 3 m column.

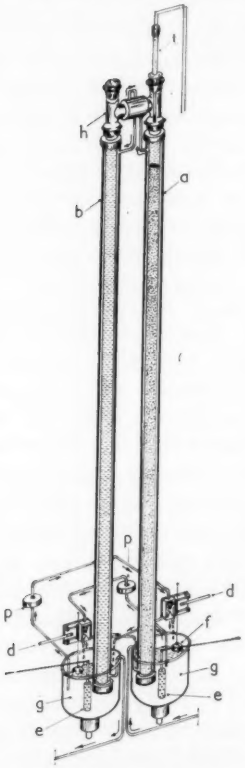

Fig. 1. Electrophoresis column for separation of nucleic acids from proteins in cellular extracts, and for the separation of nucleic acid-free extracts

(a) Cellulose-filled Pyrex glass tubing jacketed with Perspex sleeve. The sleeve is attached to the column by compressed rubber rings
(b) Electrode limb filled with buffer solution
(d, f, p) Apparatus for circulating buffer between the electrode vessels (g)
(d) Balance regulated by means of floats (f) to maintain level in electrode vessels when exchanging buffer (continuously) between electrode vessels
(e) Platinum wire electrodes enclosed in perforated plastic wound around a plastic support.
(f) Float for level-maintaining balance (*cf.* (d))
(g) Plastic electrode vessels
(h) Connection between electrode and column limb of apparatus. Note that connection between this part and glass parts of column and electrode limb consists of semi-ball ground glass joints. During runs, upper openings are closed by greased rubber stoppers
(t) Contact thermometer that switches off current *via* a relay if temperature in column rises too high

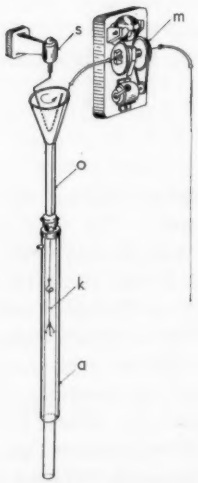

Fig. 2. Packing device mounted for packing the column with ethanolized cellulose

(a) *Cf.* (a) Fig. 1.
(k) Whisker. Note that its upper part is formed like a spiral almost touching glass walls
(m) Differential for rotating and pulling whisker upwards during packing
(o) Funnel containing cellulose-buffer slurry
(s) Motor driving a whisker to keep suspension homogenized

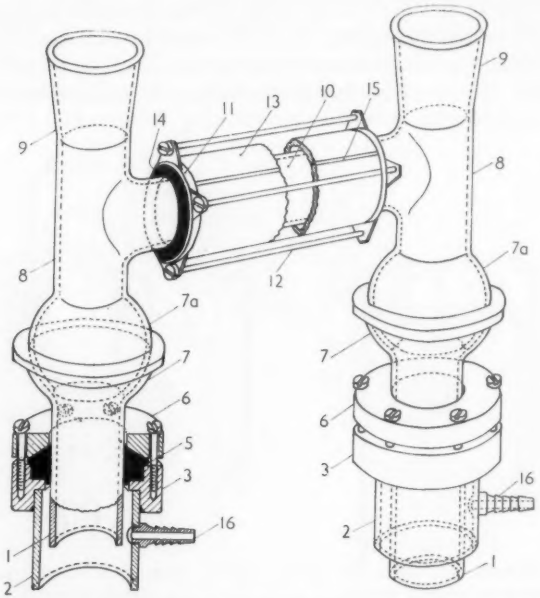

Fig. 3. Part connecting column and electrode limb during electrophoreses

(1) Glass tubing, ending in semi-ball ground joint (7)
(2) Perspex sleeve
(3) Fixed upper end of cooling sleeve
(5) Rubber ring, pressed against (1) by parts (3) and (6)
(6) Loose upper part of tightening device for pressing rubber ring (5) against (1) and (3)
(7, 7a) Semi-ball ground joint
(8) Ascending part of column-electrode limb connection
(9) Ground joint for rubber stopper and contact thermometer, respectively
(10) Glass tubing connecting 8s
(11) Device for pressing and tightening folded rubber tube against glass and metal sleeve (13)
(12) Screw
(13) Metal sleeve
(14) Double-folded rubber tube
(15) Solder
(16) Connection for rubber tubing to cooling system

The apparatus consists of an inverted U tube, from which the top can be taken off (Fig. 1). One tube (the column) is filled with ethanolized cellulose,* and the other (the side tube) with the appropriate buffer solution. The bottom of the column (Fig. 5) is fitted with a plastic support above which a layer of Pyrex

* J. H. Munktell's cellulose powder for preparative electrophoresis, Grycksbo Pappersbruk, Grycksbo, Sweden.

glass wool is packed. The bottom of the side tube is closed by a dialysis membrane (Fig. 4). The length of the column is 3·2 m and the inner diameter is 3·6 cm. The column, the side tube, and the part connecting them are all jacketed with plastic jackets and cooled with running water of +1°C. The

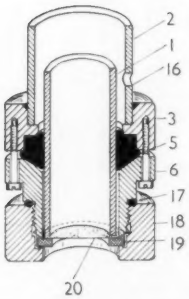

Fig. 4. Bottom of electrode limb (dialysis membrane holder)

(1, 2, 3, 5, 6) *Cf.* Fig. 3
(16) Hole for connection of rubber tubing (cooling system)
(17) Rubber ring
(18) Tapped Perspex piece to be screwed on (6)
(19) Flat rubber ring
(20) Dialysis membrane

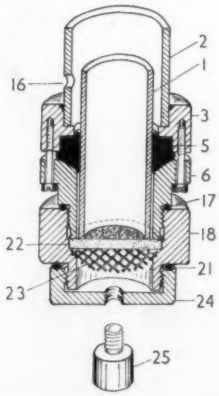

Fig. 5. Bottom of column (perforated plastic support)

(1, 2, 3, 5, 6, 17, 18) *Cf.* Fig. 4
(21) Rubber ring
(22) Part of packed cellulose
(23) Perforated plastic support
(24) Bottom plate, used between the runs
(25) Tightening plug

connection between the jackets and the glass tubing has caused trouble; it should be able to resist high voltages and not cause breakage of the glass. The final construction, which is shown in Figs. 4 and 5, has resisted 7,500 V.

Very thick-walled rubber tubing must be used for the cooling system and auxiliary apparatus in the electrode baths in order to prevent spark formation between the different parts of the apparatus.

The electrode baths, with a volume of 25 l. each, are cooled by ice-water circulating in coiled glass tubing and have apparatus that exchanges continuously the buffer between them in order to prevent changes in the hydrogen ion concentration resulting from

electrolysis. The apparatus (Fig. 6) is constructed so that the levels in the baths are kept constant in spite of different pumping rates. During the exchange the fluid enters each bath drop-wise to prevent short-circuiting of the electrodes.

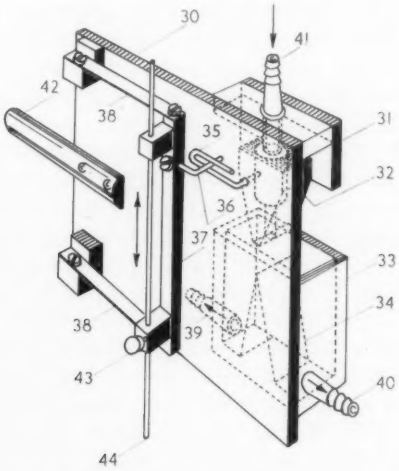

Fig. 6. Balance for maintaining levels in electrode vessels during continuous buffer exchange

(30) Plastic frame
(31) Chamber wall
(32) Balance wedge. Receiver is indicated in its upper part. From receiver, holes are bored to bottom edge of wedge
(33) Wall and chamber (*cf.* (31))
(34) Bottom wedge. Divides stream of buffer running through (34). If level in electrode vessel is too high, surplus runs back to other vessel *via* (39). At beginning of run, upper wedge (32) is adjusted to deliver half its output *via* (39) and half its output *via* (40). From (40), buffer *drips* into electrode vessel above which it is placed
(35, 36) Flexible joint connecting balance wedge and float holder.
(37) Plastic rod suspended in springs (38), holding stainless steel stave (44) fixed to float.
(38) Stainless steel springs
(39, 40) Outlets described under (34)
(41) Inlet for buffer. The buffer is transported to this inlet from the other electrode vessel *via* a membrane pump. This pump is indicated in Fig. 1 (p)
(42) Holder for use with common laboratory clamps
(43) Screw fixing (44)
(44) Stave mentioned under (37)

The electrodes consist of platinum–iridium wiring wound on plastic rods, and voltage is supplied by a conventional rectifier with a large capacitor (to prevent variations in the voltage supplied to the column). It is built to supply 7,500 V and 1·5 A.

A contact thermometer fitted in a rubber stopper is inserted in

the column, touching the upper surface of the cellulose; it is connected to a circuit breaker that disconnects the high-voltage supply when the temperature rises above a pre-set value (+10°C).

Filling the side tube is facilitated if a thick dialysis membrane is inserted at its bottom; it is then possible to pour in the buffer solution from above, the top of the side tube being then closed by a rubber stopper. Because some of the components in the extracts have a pK value higher than 7·4, which is the pH at which most of the electrophoreses were run, these substances will migrate towards the negative pole and into the side tube when the electrophoresis is run with the column at the positive pole. If the side tube was not closed by a dialysis membrane these substances would migrate *via* the buffer pumps into the column, contaminating the substances running towards the positive pole.

Usually 1 g (dry weight) of the sample, dissolved in as small a volume as possible, is introduced at the top of the column.

A pH of 7·4 was chosen in the electrophoretic runs as it seemed to give the best separation of nucleic acids from proteins. Preliminary experiments with paper electrophoresis showed that it was possible to use a higher pH (8·6), which makes all the proteins migrate towards the positive pole. When tried on the electrophoresis column, however, the rear part of the nucleic acids came out mixed with the front of the proteins. Runs at pH 8–8·1 seem to give the best separation, and the proteins and the acid polyelectrolytes are as well separated at pH 8 as at pH 7·4.

The voltage used, in most instances, has amounted to 5 V per cm, i.e. about 3 kV between the electrodes. Higher voltages have been tried, especially when borate at pH 8·6 was used, but the resolution was less marked, probably due to heat convection in the column. Lower voltages do not seem to offer any advantage. The current in 0·05 molar phosphate at pH 7·4 ($\mu \simeq 0{\cdot}15$) was 0·15 A at 5 V/cm^{-1}.

The electrophoreses have been run for 74–94 h. The mobility of the front of the nucleic acids is 3·3 cm/h at pH 7·4 in 0·05 molar phosphate buffer. These times correspond to a utilization of 76–97 per cent of the column height.

Some experiments have been performed in which only part of the column was utilized. When half or two-thirds of the column was utilized, the rear fractions of the nucleic acids came out

together with the front of the proteins (0·05 molar phosphate, pH 7·4). We were thus forced to use practically the total height of the column.

The column is eluted with de-aerated buffer after the electrophoresis. The eluate is sampled in a fraction collector cooled with running ice-water. The recommended volume per tube is 5–10 ml. With the packing technique to be described the elution rate is about 0·4 ml/min.

Filling the Column with the Solid Supporting Medium. The resolving power of an electrophoretic column depends mainly on the homogeneity and tightness of the supporting material. This material has in all cases been cellulose, made more inert by esterifying the polar groups with ethyl groups.

It is not difficult to achieve ideal packing of a column that is only 50 cm long, but the difficulty increases out of all proportion with increasing length. Nevertheless a column 320 cm long had to be used to achieve the all-important separation of acid polyelectrolytes (especially the nucleic acids) from the proteins.

The cellulose powder (1·75 kg) is suspended in 20 l. of 0·05 molar phosphate of pH 7·4. The suspension must be freed of air. The temperature of the suspension is then adjusted to that inside the column. No change in temperature should be allowed in the room, and in particular, no temperature gradients—the opening of a window, for example, may completely destroy packing that is under way. The cooling jacket should be emptied the day before a packing is planned. When the packing is completed it is not sensitive to uniform changes in temperature and consequently it does not react unfavourably to the lowering of the temperature occurring when cooling water is admitted to the jacket ($+1°C$), or by the subsequent passage of an electric current, which raises the temperature to 8–9 °C when 450 W are dissipated in the column.

The cellulose powder is microscopically inhomogeneous as to particle size. Material sifted to sizes between 200 and 400 mesh was tried but no advantage was found when using this more homogeneous powder. In short columns it was found that a suspension of cellulose powder in 96 per cent ethanol settled well, and that by gradually substituting the ethanol with phosphate buffer a seemingly very good packing could be achieved. When this

method was applied to the 320 × 3·6 cm column the result was not satisfactory.

Auxiliary devices for packing the column. The suspension was poured into the column through a funnel 150 cm above the column top which was kept full of suspension during the packing process. The suspension was vigorously stirred by a suitably bent glass rod. This homogenizer suffices for short columns but is not satisfactory for a 320 cm packing. Preliminary experiments showed that the formation of lacunae could be prevented by gently stirring the suspension in the region about 1 cm above the top of the settled cellulose, and a special device was constructed to effect this stirring (Fig. 2). A sweep of stainless steel wires was suspended on a 5 m length of tightly wound stainless steel spring coil. This could be rotated and moved upwards or downwards by means of the differential shown in Fig. 7. In this way the sweep could be kept in slow rotation about 1 cm above the packing zone during the packing process.

The cellulose powder settled at 0·25–0·35 cm/min, depending on the thickness of the suspension. This could not be kept constant throughout the unpacked region and was therefore siphoned off and re-mixed twice during each packing cycle, i.e. every fourth or fifth hour. It is permissible to stop the packing half-way through (7–8 h) and leave the column under a hydrostatic pressure of buffer overnight. In the morning about 10 cm of the cellulose top layer is stirred up by moving the sweep in rapid rotation downwards before packing is resumed.

Buffer solution escapes continually through the bottom of the column. The flow rate and the hydrostatic pressure both influence the packing. The flow through the column, of course, decreases with increasing length of packed cellulose, and during the packing of the first metre the flow can—and should—be decreased to 5 ml/min. Then it begins to decrease down to 1–2 ml/min at full packing plus a hydrostatic pressure of 60 cm above column top. When the packing is finished the column is eluted overnight with buffer. Then 0·5 l. of 0·1 molar sodium hydroxide in water is passed through and removed by the usual buffer solution. After that the column is tested for homogeneity by passing a band of phenol red down it. The whole packing process including the testing of the column takes about 7 days, and the outflow is 1 ml/min.

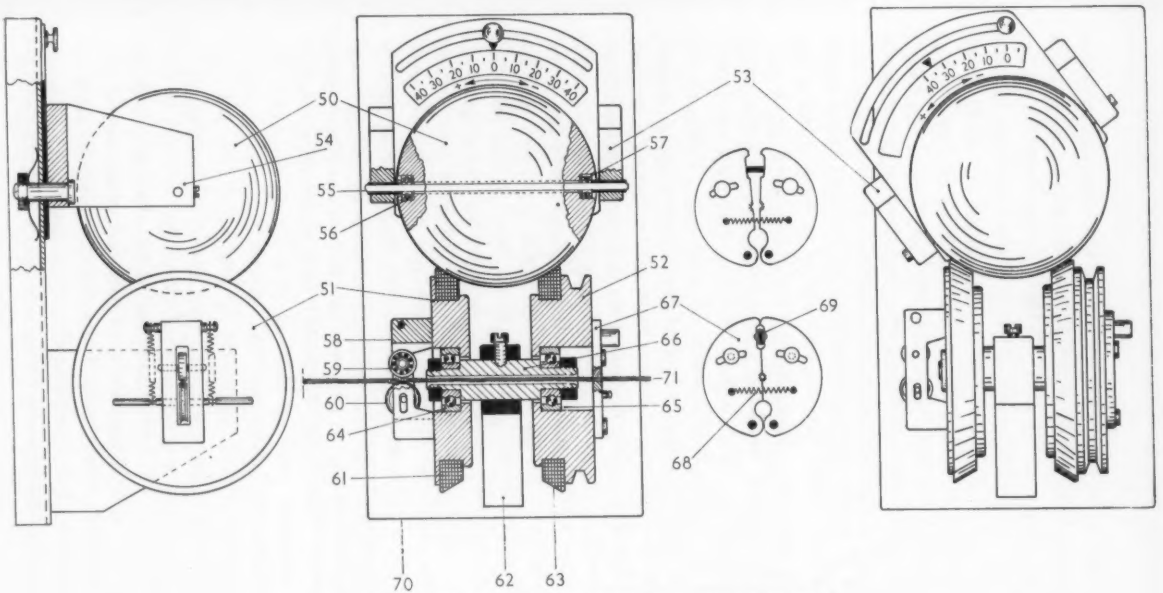

Fig. 7. Differential mechanism for packing whisker

(50) Plastic sphere
(51, 52) Bakelite wheel
(53) Bearings for sphere, attached to support by means of regulating screw
(54) *Cf.* (53)
(55) Axis for plastic sphere. Preferably supported by ball bearings
(56) Interior fixation of plastic sphere to axis
(57) *Cf.* (56)
(58) Support on wheel for device rotating spiral (71)
(59) Ball bearing fixing spiral
(60) Ball bearing pressed against spiral by means of spring on its axis
(61) Rubber with roughened surface
(62) Support holding hollow axis of wheels
(63) *Cf.* (61)
(64, 65) Ball bearings between axis and hollow axis and wheels
(66) Hollow axis
(67) Split nut for moving spiral in its longitudinal direction
(68) Spring for holding split nut together
(69) Dowel pin
(70) Support
(71) Stainless steel spiral

Tight packings give better resolution of the components, and different packing techniques were tried to obtain such tight columns. We found: (1) tightness of the column depends on the amount of cellulose powder that goes into the packing, (2) this amount may be increased by using a relatively high hydrostatic pressure during the packing process, (3) the glass of the column should be carefully inspected where tube sections have been sealed together, for the presence of even the slightest circular narrowness. This will cause very bad spreading and must be corrected.

On the basis of these observations some alterations in the auxiliary apparatus were made for the purpose of getting more cellulose settled per 320 cm. The sweep was substituted by a thin plastic disc, weighted on its upper surface with a 150 g elongated lump of stainless steel. The disc diameter was 30 mm and it was perforated by as many 2 mm holes as possible. Between the differential gear and the column to be filled the spring coil passed over a lever, the movements of which were controlled by a maltese cross with ten 'spokes'. By means of an indented plastic wheel, coupled to the maltese cross, the lever quickly lowered the disc once a minute onto the settled cellulose. It remained there for a few seconds and was then slowly drawn up again to about one cm above the packed cellulose. During these movements the disc rotation was stopped by means of a microswitch arrangement. When the disc rotated above the settled cellulose it was rattled three times per cycle to shake off cellulose that might have settled on its upper surface. In its present shape the apparatus has to be watched continually, but it can easily be automatized by using the slackening of the spring coil when the plastic disc is let down. With this improved technique reproducibly homogeneous columns with an outflow of 0·4 ml/min were obtained.

Chromatography

The fractions from an electrophoresis were concentrated by ultra-filtration to small volumes and diluted with water to 0·02 molar phosphate for chromatography on DEAE (and in some instances CMW).[4, 5] In many instances the fractions were dialysed against the appropriate buffer.

Discussion

We have been engaged mainly in trying out methods for resolving whole cell extracts of *B. pertussis* to yield material for the team of microbiologists to test for immunological and toxic activities. From a biochemical point of view, material has been scarce during this phase of the work. But recently the microbiologists succeeded in making 500 l. cultures with a population of 35×10^9 per ml of highly virulent *B. pertussis* and this will furnish enough material for electrophoretic runs on the higher capacity column ($320 \times 7{\cdot}5$ cm and capacity for 10 g solids) which is now ready for use.

Methods of extraction have been changed during the work and only gradually did we find that we must change over from freeze-drying to ultra-filtration in order to avoid denaturation. For these reasons chemical analyses of the fractions are as yet incomplete and are withheld for a later communication.

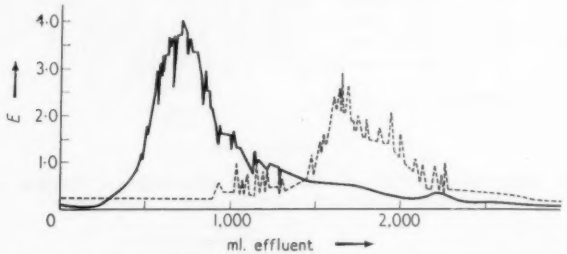

Fig. 8. Loose, unsatisfactory packing of column. Elution rate: 2 ml/h. Solid line depicts extinction at 260 mμ; broken line, extinction by ninhydrin reaction at 570 mμ after hydrolysis with HCl to amino acids. For further details see text

Figs. 8–10 are given as a demonstration of results before ultra-filtration was introduced. Repeated lyophilizing had been used for preparing the cell extracts (*B. pertussis*) for the electrophoretic runs in Figs. 8 and 9, both runs being made in 0·05 molar phosphate at pH 7·4 and 5 V/cm.

It is necessary to use the total length of the column to get a satisfactory separation of protein-free acid polyelectrolytes ('nucleic acid fraction'). This was shown in other runs where only $\frac{1}{3}$–$\frac{1}{2}$ of the column was used electrophoretically. The mixing

between the rear part of the 'nucleic acid fraction' and the front fraction of the proteins was increased in inverse proportion to the effective length of the column.

Some other factor or factors must be involved because the runs represented in Figs. 8 and 9 both corresponded to an effective length of about 80 per cent of the total length, and Fig. 8 (loose

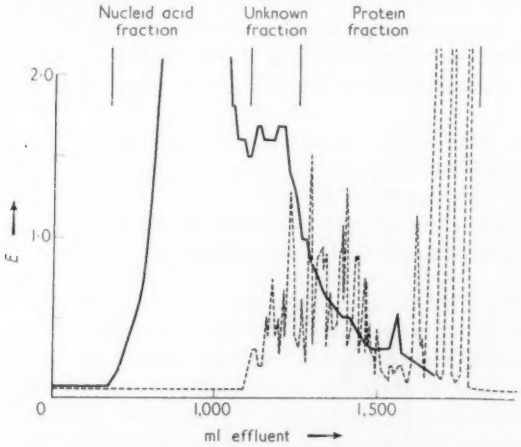

Fig. 9. Very good packing of column (not reproducible). Elution rate: 0·4 ml/min. For further details see Fig. 8 and text

packing) shows a much better separation of the two main fractions than Fig. 9 (tight packing).

We have found that one cannot attain all aims at one pH. At pH 7·4 an important fraction* of proteins with a pK 7·4 migrates towards the cathode and is lost in anodic runs.

When runs were made in borate buffer at pH 8·6 this fraction migrated towards the anode but then the mixing of 'nucleic acid fraction' and proteins was pronounced.

Runs are now made at pH 8† for the isolation of a neurotoxic

* The fraction has neurotoxic activity and contains a protein that is dark blue in the oxygenated form.

† Recent experiments, show that the neurotoxin loses its toxic activity in two weeks at pH 8 and pH 6 at 0°C, but is more stable at pH 7·4.

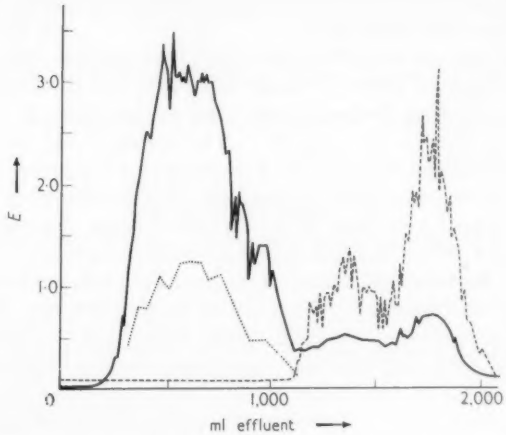

Fig. 10. Reproducible standard packing. Elution rate: 0·4 ml/min. Dotted line indicates extinction of purines isolated after hydrolysis of 'nucleic acid fraction'. Figure shows that amount of purines approximates zero when the proteins begin to emerge from column

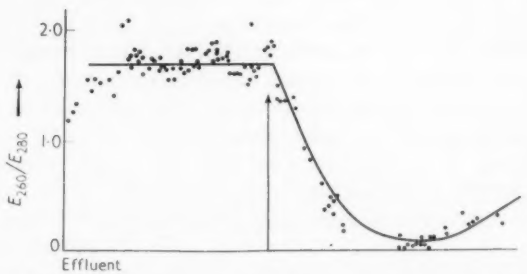

Fig. 11. Relation of extinction at 260 and 280 mμ in range between acid polyelectrolytes and proteins. Ratio was computed from experiment described in Fig. 10. Arrow indicates position where 1,100 ml had been eluted from column. At this position final traces of 'nucleic acids' had been eluted and first proteins were being eluted. Quotient 260/280 is typical of nucleic acids up to arrow after which it rapidly declines. Curve displays good separation of nucleic acids from proteins. For further details see text

protein fraction and will be made at pH 6 for separations within the 'nucleic acid fraction'.

Most of the material from the analytical column (320 × 3·5 cm) has been used for activity tests. The ratio of light absorption at 260 mμ to that at 280 mμ in the first region, from some runs at pH 7·4, is recorded in Fig. 11. The sudden drop in this ratio coincides with the appearance of peaks giving a positive ninhydrin reaction increasing after hydrolysis, clearly indicating proteins. The analysis for purines (Fig. 10) shows that the nucleic acids were eluted before the first protein fractions appeared. We have tried to detect the first appearance of amino acids in this region by paper chromatography. Products of acid hydrolysis of the non-protein components, however, give disturbing reactions. This makes the detection of minute amounts of amino acids difficult if not impossible.

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Control of Foam during Fermentation by the Application of Ultrasonic Energy

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Summary. An ultrasonic generator was tested at 3 frequencies for its effects on *Serratia marcescens* during fermentation. The generator controlled foam adequately at 26, 29, and 34 kc/sec at medium aeration rates up to 0·6 ft^3/min. Final viable counts were in the same range as those for cultures grown using lard oil as a foam control agent. At 34 kc/sec the final viable counts were slightly higher than those of cultures with lard oil.

No harmful effects were evident when large volumes of cultures were exposed to ultrasonic energy during growth.

Introduction

Actively aerated fermentations invariably produce foam which can foul the apparatus and cause other problems in handling. The stability of the foam generated depends upon the surface tension, viscosity, pH, and the concentration of the solutes in the medium.[1] Chemical anti-foam agents have been employed successfully in foam control. There are situations, however, in which chemical anti-foam may adversely affect further processing steps. For example, traces of anti-foam remaining as residue may adversely affect steps such as centrifugation, filtration, and drying; therefore, a physical means, such as ultrasonic vibration, is most desirable.

It is known that ultrasonic vibration, if sufficiently intense, will disintegrate foam.[1] The present study was undertaken to obtain data on the application of ultrasonic vibration to the control of foam during the growth of *Serratia marcescens*.

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Materials and Methods

The ultrasonic generator selected for application to the problem of foam control was based on the design used by Hartmann.[2] The aluminium generator (Figs. 1 and 2) had a jet 0·078 in. in diameter,

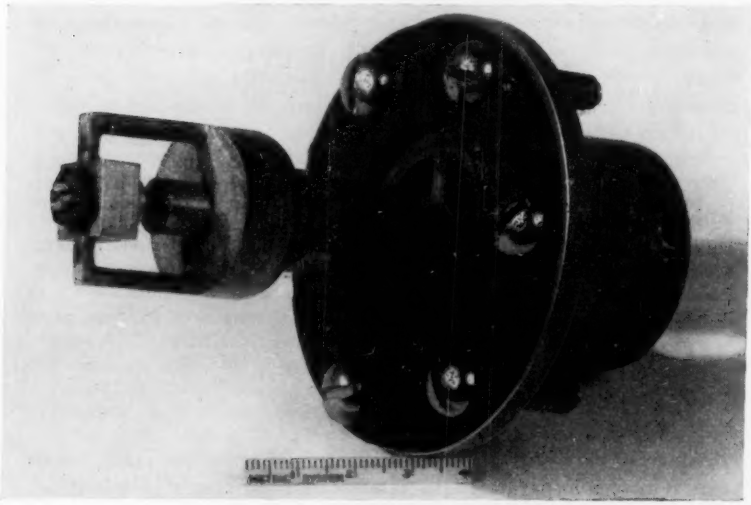

Fig. 1. Air-driven ultrasonic generator

and a cavity 0·078 in. in diameter and 0·078 in. in depth. The generator is simple in construction, has no moving parts, and when incorporated in fermentors is easily sterilized. The generator was tested at 26, 29, and 34 kc/sec. A minimum pressure of 40 lb/in^2 (gauge) and a free air-flow rate of 2 to 4 ft^3/min were required for satisfactory operation. The generator was mounted in a Plexiglass lid that fitted the top of the fermentor. Use of the Plexiglass lid permitted observation of the tank's interior during the process of fermentation. A vent was built into the lid to allow for release of air from the fermentor to the atmosphere. If the generator is installed in fermentors, the vent capacity would have to be increased to remove the additional air used to drive the generator.

Incoming air was filtered through two filters, each packed with

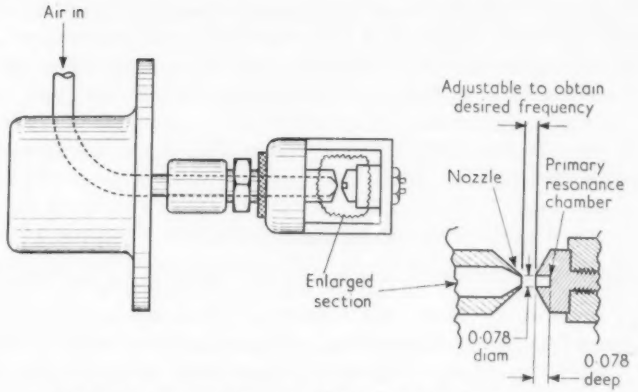

Fig. 2. Schematic drawing of air-driven ultrasonic generator

resin-impregnated glass-wool mats (see Fig. 3 for schematic diagram).

The organism used was *Serratia marcescens*, strain 8UK.

Casein acid digest (CAD) medium was used in these experiments. The complete medium consisted of: casein acid hydrolysate, 10 per cent; plasmolysed yeast (dry basis), 0·5 per cent; cerelose,

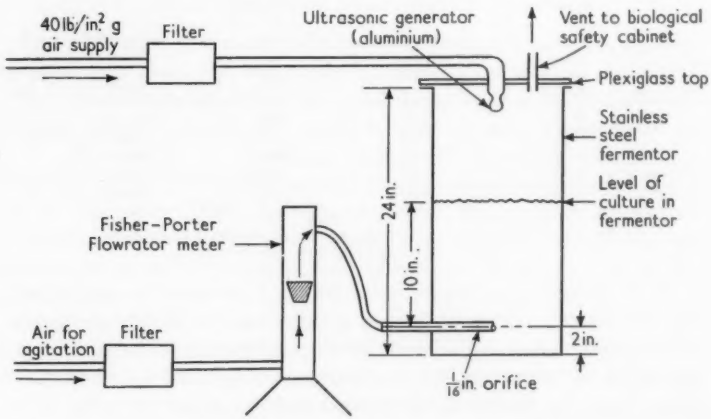

Fig. 3. Schematic drawing of equipment

0·75 per cent; K_2HPO_4, 0·5 per cent; thiamine hydrochloride, 0·0025 per cent; $MgSO_4 \cdot 7H_2O$, 0·0049 per cent; $MnSO_4 \cdot 4H_2O$, 0·0022 per cent; and $FeSO_4 \cdot 7H_2O$, 0·0028 per cent. The pH of the medium was adjusted to 7·0–7·2 before sterilization. All cultures were grown at 25°C.

All inocula were grown in the casein acid digest medium described above for 18 h at 25°C.

Experimental Work

Effect of Aeration Rates on Foam Control by Generator

In preliminary tests, the ultrasonic generator's capacity to suppress foam was evaluated on casein acid digest medium without living organisms. Results of these tests revealed that the effectiveness of the mechanical foam-control unit depended chiefly on the unit's distance from the surface of the liquid and the medium aeration rates. The generator used in these experiments was most effective at a distance of 8 in. from the surface of the liquid (see Table I).

Table I. Aeration rates *versus* generator foam control at 29 kc/sec

Superficial air velocity ft/min	Vol air/vol liquid, min	Aeration rates SCFM	Foam breakage
0·25	0·18	0·2	Good
0·38	0·26	0·3	Good
0·51	0·35	0·4	Good
0·64	0·44	0·5	Good
0·77	0·53	0·6	Good
0·89	0·62	0·7	Fair
1·05	0·71	0·8	Poor
1·15	0·79	0·9	Poor
1·28	0·88	1·0	Poor

Volume—22 l. Medium used—Casein acid digest. Exposure time—30 min. Generator was located 8 in. from surface of liquid.

If air in increasing amounts is bubbled through the orifice into the fermentor, a rate of flow is reached at which the generator cannot adequately suppress the foam. Foam breakage was good when the aeration rate was in the range of 0·2 to 0·6 ft^3/min. In this range, the height of the foam averaged approximately 1 in.; at 0·7 ft^3/min there was approximately 2 in. of foam on the sur-

face of the liquid; at 0·8 and 0·9 ft^3/min the height of the foam was approximately 3 to 4 in.; and at 1 ft^3/min the foam slowly built up until it reached the generator. When the foam reached this point an aerosol, created by the air-flow in the sonic field around the generator, prevented the foam from rising higher than the generator.

When the generator was operated at 26 kc/sec, which was the lowest frequency obtainable with this generator, there was little or no difference in the foam-breaking capabilities from those noted when the generator was operated at 29 kc/sec. However, there seemed to be a slightly greater cavitation of the surface of the liquid at 26 kc/sec.

There was a noticeable difference in foam-breaking ability when the generator was operated at 34 kc/sec, which was the highest frequency obtainable. The effective range of the energy field was not as wide as the diameter of the tank; the range being approximately 11 in. with about a $\frac{1}{2}$ in. area around the edge of the medium not affected. The foam in this area, instead of being destroyed, was forced up the side of the fermentor to a point halfway between the surface of the liquid and the generator.

Effects of Lard Oil and Ultrasonic Vibrations on *Serratia marcescens* Viable Cell Counts

One objection to the use of ultrasonic energy as a foam control method has been its reported detrimental effects on bacterial cells.[3] Roth *et al.* subjected live, autolysed spores of *B. anthracis* to sonic vibrations at 9,000 c/sec for various time intervals up to 1 h. Subsequent examination under the electron microscope showed no discernible effect on samples exposed to sonic vibrations up to 10 min. After 15 min exposure many cells appeared wrinkled, indicating damage to their spore coats.[4] The following trials were made to determine the effects of ultrasonic vibrations on viability.

Each culture was grown for a period of 24 h. The lard oil, 0·02 per cent by volume, was added to the fermentor at the time of inoculation. Samples were taken and viable counts made at 18, 20, 22, and 24 h. When the mechanical foam-breaker was used, the culture was exposed to ultrasonic vibrations, 29 kc/sec,

of the generator immediately after the medium was inoculated (5 per cent inoculum), the exposure continuing for 24 h. Six replicates each were run using lard oil and the ultrasonic generator. The results are recorded in Table II.

Table II. Effects of lard oil and ultrasonic vibrations on *Serratia marcescens* viable cell counts

Anti-foaming agent	Average viable cell count $\times 10^9$ per ml/h			
	18	20	22	24
Ultrasonic energy	14·0	13·0	11·0	16·0
Lard oil	15·0	15·0	14·0	15·0

There was no significant difference between the effects on viable cell recovery of either lard oil or the generator.

The effects of different frequencies of ultrasonic vibrations on the viable cell counts of *S. marcescens* as compared to those using lard oil were investigated. Samples were taken and viable counts made at 18, 21, and 24 h after inoculation. One set of replicates was exposed to ultrasonic vibrations for 24 h. The second set had lard oil, 0·02 per cent by volume, added at the time of inoculation. The viable counts of the *S. marcescens* cultures exposed to ultrasonic vibrations of 26 kc/sec, 34 kc/sec, and cultures grown using lard oil as an anti-foam agent are shown in Table III.

There is no significant difference between viable counts of cultures exposed to 26 and 34 kc/sec and viable counts of cultures grown using lard oil as anti-foam agent.

Table III. Viable cell counts $\times 10^9$ per ml of *Serratia marcescens* exposed to ultrasonic energy and lard oil

Age of culture[a]	Ultrasonic generator, 26 kc/sec	Lard oil	Ultrasonic generator, 34 kc/sec
18	15·0	15·0	15·0
21	14·0	15·0	18·0
24	16·0	15·0	17·0
Average	15·0	15·0	17·0

[a] Hours after inoculation.

Discussion and Conclusions

Under the conditions of these experiments it is evident that bacteria exposed to ultrasonic energy were not killed. This may be attributed to many factors including the volume of the medium used and the distance that the generator was located from the surface of the liquid. The most important factor, however, is the poor acoustic coupling between air and water. Because the impedance discontinuity is very great at the air–water interface, almost all of the airborne energy from the generator is reflected. Only a small fraction of the ultrasonic energy enters the liquid.[5]

The preliminary data reported here indicate that use of the ultrasonic generator is an effective method for control of foam in fermentors. It compares favourably with foam control by use of chemical anti-foam. Important among the advantages of a mechanical method over the use of chemical additives in foam control are: (1) possibility of operations with greater volumes of cultures, and (2) application without regard to final processing steps.

Acknowledgements. The author wishes to thank Warren W. Bowden for his help in formulating the plan and design of the equipment used in this work, and Mr. Charles R. Moberly, III, for fabricating both the ultrasonic generator and fermentor. Thanks are also due to Mr. John S. Derr, Jr., for calibrating the ultrasonic generator. Messrs. E. R. Dapper, R. Smith, and John Lemp, Jr., were especially helpful in their constructive criticism of part of this work.

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Automatic Feed Device for Laboratory-size Glass Circulating Evaporator

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Summary. A simple, rugged and compact automatic feed control attachment for the laboratory-size glass circulating evaporator has been designed and constructed. Experience has demonstrated that this apparatus operates satisfactorily with a variety of aqueous biological extracts and solutions having resistivity values of 50,000 ohm-in. or less. The device is adaptable to other evaporators and stills.

Introduction

The problem of reducing to a small volume a large volume of aqueous solution which contains biologically active materials is a frequent occurrence in the isolation of natural products. The evaporation is difficult whenever the substances to be isolated are sensitive to heat, and under these conditions the water must be removed under reduced pressure at low temperature. In this laboratory the concentration of bacterial extracts containing colominic acid,[1] a heat sensitive polymer of *N*-acetylneuraminic acid, from 15–35 l. of fluid to a 2–3 l. volume is a routine procedure. For this purpose a glass circulating evaporator[2] operated at 8–12 mm pressure and a temperature of 18–30°C is currently employed. However, to maintain the small hold-up volume of approximately 3 l. contained within the evaporator under normal operating conditions, frequent addition of fresh fluid is necessary, thus requiring considerable attention by a technician. This paper describes a convenient and simple automatic feed control

device which permits addition of extract to the still and maintains the fluid level in the evaporator to within a few 100 ml fluctuation. The automatic feed device embodies an additional novel control feature in that air cannot be drawn into the evaporator once the liquid in the reservoir is depleted.

Automatic Feed Control Unit Description

Fig. 1 is a diagram of the laboratory-size glass circulating evaporator with the automatic feed control attachment. This device consists of four parts. Unit A contains the electrical circuits and has three toggle switches S_1, S_2 and S_3 and two

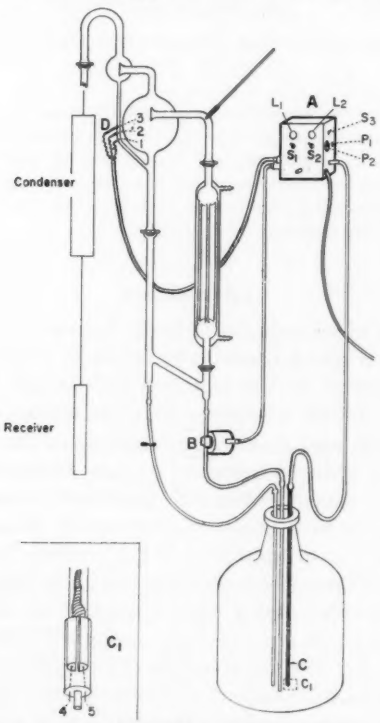

Fig. 1. Laboratory-size glass circulating evaporator with automatic feed control attachment

potentiometers P_1 and P_2; S_1 is a power switch; S_2 is an automatic manual switch—part of the level controller circuit which maintains the fluid level within the evaporator; S_3 is part of the reservoir controller circuit which prevents air from entering into the evaporator if the fluid in the reservoir becomes depleted. Unit B is a stainless steel solenoid valve through which fluid passes from the reservoir to the evaporator. Unit C, the reservoir controller unit, consists of two copper wires contained within a polyethylene tube and connected to platinum wire electrodes 4 and 5. Unit D consists of three platinum wire electrodes 3, 2 and 1 located at 12, 14 and 16 cm below the small bowl of the evaporator.

Operating Instructions

Level controller. To operate the level controller, turn potentiometers P_1 and P_2 (Fig. 1) to the extreme anti-clockwise position. Set the reservoir switch S_3 to the off position—this step removes the reservoir controller circuit from the level controller circuit. Set the automatic manual switch S_2 to automatic. Turn the power switch S_1 to the on position. Both the power indicator lamp L_1 and solenoid indicator lamp L_2 should be lit and fluid should now be filling the evaporator. When the fluid reaches electrode 3, rotate the level potentiometer P_1 clockwise (screwdriver adjustment) slowly until the solenoid closes, which is indicated by light L_2 going off. The level control system should now operate automatically for fluid. A clockwise rotation of the level control potentiometer an additional 5 or 10° should allow the level control system to operate on foam. This adjustment varies with the conductivity of the fluid.

Reservoir controller. To operate the reservoir controller, rod C is inserted into the reservoir. While the solenoid valve B is open, indicated by L_2 being lit, the reservoir switch S_3 is set to the on position. With the potentiometer P_2 set at zero, light L_2 should go off. Rotate the potentiometer knob P_2 clockwise until light L_2 illuminates and solenoid valve B opens. Advance knob P_2 an additional 5° beyond this point. The solenoid valve B now will close if the fluid in the reservoir breaks contact with electrodes 4 and 5. This action can be tested by withdrawing rod C from the reservoir while the solenoid is open.

Circuit Description

The two functions of the control unit are to maintain the fluid level in the evaporator between two fixed limits and to close the intake line when the reservoir is empty. Although the unit operates best with fluids having low resistance, satisfactory operation may be obtained with fluids having resistivity values up to 50,000 ohm-in.

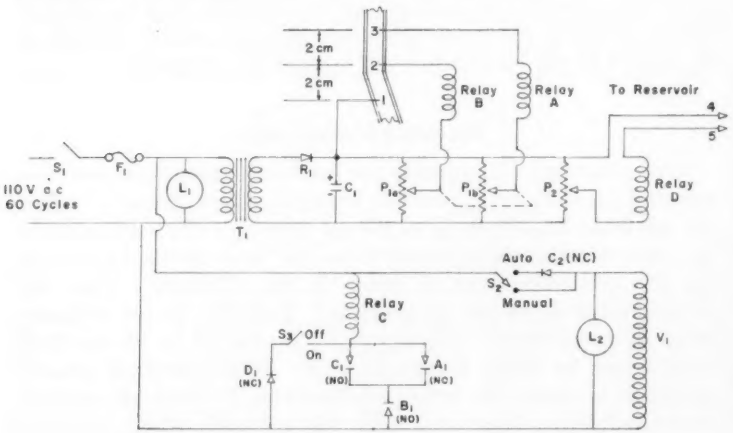

Fig. 2. Wiring diagram of feed and reservoir controllers

C_1	20 μF 150 V electrolytic capacitor	C	110 V, 60 cycle relay, double-pole double-throw contacts
F_1	1 A fuse	S_1, S_3	single-pole single-throw toggle switches
L_1, L_2	110 V, 6 W lamps	S_2	single-pole double-throw toggle switch
P_1 (P_{1a} P_{1b})	dual 20,000 Ω, 4 W potentiometers	T_1	110 V/110 V, 60 cycle transformer (100 mA)
P_2	20,000 Ω, 4 W potentiometer	V_1	110 V, 60 cycle solenoid valve, normally closed
R_1	1N92 rectifier cell	1,2,3,4,5	platinum wire electrodes
A,B,D	2000 Ω, 3 mA sensitive relays, single-pole double-throw contacts		

A diagram of the circuit and the characteristics of its components is shown in Fig. 2. When the fluid connects the platinum electrodes 1 and 2, relay B is energized, closing the normally open contact B_1. When the fluid reaches electrode 3, relay A is energized, closing contact A_1. The circuit through the coil of relay C is now complete; contact C_2 is open and C_1 is closed. Up to this point the solenoid valve has been energized and open.

When contact C_2 opens, the solenoid valve closes and remains closed so long as this contact remains open. Contact A_1 opens when the fluid ceases to make contact with electrode 3. Relay C remains energized, however, through its own contact C_1. When the fluid falls below electrode 2, contact B_1 opens the coil circuit of relay C and allows contact C_2 to close. This action closes the solenoid circuit and opens the solenoid valve to allow more fluid to enter the system. Potentiometer P_1 permits the operator to select a voltage sufficient to operate relays A and B through the resistance of the fluid but insufficient to operate these relays through the resistance of the wet tube walls.

The reservoir control circuit operates relay D. The level control circuit operates as discussed above when potentiometer P_3 is adjusted so that sufficient current flows through the fluid between electrodes 4 and 5 to energize relay D, thereby opening contact D_1. When the fluid level falls to a point where low resistance contact between electrodes 4 and 5 no longer exists, contact D_1 closes, and relay C is energized causing the solenoid valve to close.

Transformer T_1 isolates the control circuits from the 110 V line. The rectifier and filter condenser provide a d.c. voltage for the sensitive relays. Switch S_2, when in the manual position, causes the valve to remain open. Switch S_3, if thrown to the off position, permits the level control circuit to be used without the reservoir control feature.

Discussion

Aqueous extracts of biological origin often contain substances which result in foam formation upon evaporation of the extract under reduced pressure. Addition of an anti-foam agent such as octyl alcohol or tributyl phosphate is frequently required when conventional distillation techniques embodying a round-bottomed flask as the basic apparatus are employed. Evaporation of extracts in the circulating evaporator considerably reduces the problem of foam formation and the addition of anti-foam agents is not usually required. However, under manual operation, the too rapid addition of fluid to the evaporator when under reduced pressure often results in excessive foaming and the spilling of

fluid into the distillate. The automatic feed control device eliminates these problems by permitting a slow steady addition of extract into the system.

Operation of the evaporator under atmospheric conditions requires positive air pressure applied against the fluid in the reservoir so as to maintain a pressure head against the solenoid valve for effective fluid intake into the system. The pressure head, obtained by raising the reservoir slightly above the fluid level in the evaporator and siphoning the liquid from the reservoir by gravity, is adequate for effective feeding of fluid through the solenoid valve.

Once the automatic feed control device is set to operate, no further attention is required during the entire period of the evaporation process. In this laboratory the control device has operated over a period of 12 h without attention.

The control apparatus is compact, rugged and does not require frequent re-adjustments due to drift or changes in solution conductivity. Solutions with a resistivity of 50,000 ohms-in. or less can be fed satisfactorily into the evaporator by the automatic feed controller.

The reservoir controller eliminates the intake of air into the evaporator with its subsequent problems of violent bumping and, in some circumstances, loss of biological activity.

The instrument has been in use for several months in this laboratory and has operated satisfactorily during this interval. The apparatus, with slight modification, can be used in conjunction with other evaporators and stills. The feed control device is highly recommended for use in laboratories engaged in the concentration of plant or bacterial extracts, urine or when water must be removed by evaporation under controlled conditions.

Acknowledgement. The authors gratefully acknowledge the co-operation and assistance generously given by the South-eastern Scientific Company of Knoxville in the construction of this apparatus. This work has been supported by a grant from the U.S. Public Health Service.

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A Method of Achieving Photosynthesis in a Stainless Steel Fermentor

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Summary. A description of the cultivation of *Rhodospirillum rubrum* in 1,000 and 3,000 l. fermentors fitted with a special illumination device, and a discussion of the results.

Introduction

Current interest in microbial photosynthesis frequently causes a need for large quantities of cell material which can be used for chemical studies of the process. Many special devices have been described, particularly for mass culture of algae (Myers *et al.*;[1,6] Burlew;[2] Gummert, Meffert and Stratmann;[3] Tamiya *et al.*;[4,7,8] Krauss and Thomas;[5] Kanazawa, Fujita, Yuhara and Sasa;[9] Enebo;[10] Fogg, Smith and Miller[11]), but to our knowledge only Preuss *et al.*[12] have used ordinary closed fermentors for photosynthesis work. They illuminated the culture through a window on the tank, a method which, however, has some drawbacks. The purpose of the present paper is to describe the use of a lamp inside a fermentor where the culture is given a large surface in a vortex formed by a bottom stirrer (*cf.* Chain *et al.*[13]). The technique has been worked out for *Rhodospirillum rubrum*, but we feel that the ease with which an ordinary fermentor is adapted to photosynthesis might provide useful knowledge also for those working with other types of microbial photosynthesis.

Background to the Experiments on *R. rubrum*

As a consequence of the studies by Vernon, Kamen *et al.*[14–17] in particular, a great deal is now known about the metabolic

pathways in *R. rubrum*. The material investigated has largely been obtained from old cultures grown anaerobically under strong illumination. The authors mentioned have recently focused their attention on the Red Heme Protein (RHP) and certain studies of cytochrome c have been performed at the Nobel Institute in Stockholm on material which they have kindly provided (Paléus and Tuppy[18]). In the course of those investigations it soon proved necessary to use large batches of cell material and part of the cultivation equipment available at the Bacteriological Department[19] was then temporarily adapted for this purpose.

Medium

The medium used had the following composition:

dl-Malic acid	3·5 g
l-Glutamic acid	4 g
Sodium citrate . $5\frac{1}{2}$ H_2O	0·8 g
Biotin	10 γ
$MgSO_4 . 7\ H_2O$	0·2 g
$CaCl_2$	38 mg
KH_2PO_4	120 mg
K_2HPO_4	180 mg
Yeast Extract Difco	0·5 g
Distilled water to make	1,000 ml

After adjustment of the pH to about 7 the medium was sterilized by autoclaving for 15 min at 120°C. For plating, etc., the same medium was used with the addition of 2 per cent agar.

Cultivation technique

Pre-cultures were grown in ordinary 100 and 1,000 ml Erlenmeyer flasks overlayered with sterile liquid paraffin. In the next step low 3 l. culture flasks,[20] fitted with rubber stoppers holding a simple lock consisting of a folded glass tube containing liquid paraffin, were used. All flasks were illuminated from a bank of electric lamps which were adjusted to such a distance that the flasks attained a temperature of about 30°C. They were then exposed to about 100 lm/l. (about half of the light flow from the lamps).

A subcultivation chain was started from a stab culture of

R. rubrum. This was used to inoculate 10 ml of medium covered by paraffin. After about a week under illumination a new step was started by transfer to a flask holding 100 ml of medium. After similar intervals further steps followed, each time with a tenfold increase in volume until finally 50 l. of culture were contained in the 3 l. flasks just mentioned. The smaller culture volumes were frequently stirred by hand, the larger ones being agitated by magnetic stirrers. Intermittent stirring was frequently found to cause formation of gas (*cf.* Gest and Kamen[21]), making the use of the gas traps previously mentioned imperative. They also ensured sterility, which is of course a factor of importance in a subcultivation chain of the length used in the experiments described. Whereas the early steps might need 7–10 days to reach the desired density, later cultures were frequently ready in 5–6 days.

After microscopic examination the 50 l. glass flask cultures were mixed in a sterile room. The mixture was contained in glass carboys fitted with the Stericonnectors described by Hedén and Malmgren.[22] They were then transported to the pilot plant and used to inoculate a 1,000 l. fermentor containing 450 l. of sterile medium under nitrogen. After a week at 30°C under a slow flux of nitrogen (about 10 l./min) and vortex stirring, limited to the point where almost no gas was beaten into the medium, the culture was transferred to a 1,000 l. storage tank filled with nitrogen. After a few hours, used to move the illuminating device from the original fermentor to a 3,000 l. fermentor containing 1,500 l. of medium and to sterilize the latter fermentor, the culture was transferred under nitrogen to this tank. Here growth was resumed under similar conditions as in the 1,000 l. fermentor and reached the stationary phase within 6 days, when the culture was harvested on a De Laval DX separator.

Illumination of Fermentors

As a consequence of the vortex stirring, the surface area in the 1,000 l. fermentor could be increased by roughly 90 per cent to about 1·1 m^2 and in the 3,000 l. fermentor by 130 per cent to about 3·5 m^2, giving area to volume ratios of 0·022 and 0·018 (cm^2/cm^3), respectively. As a comparison it can be mentioned

that those ratios for the 100 ml and 3 l. flasks were 0·66 and 0·20, respectively.

As mentioned previously the flasks were illuminated with about 100 lm/l., so a very strong lamp had to be used if intensities of the same order were to be reached in the fermentors. We decided to try flood-light lamps and successfully used both a 3,000 W and a 2,000 W lamp.[23] Lamp failures occurred occasionally and we came to prefer the 2,000 W lamp, which seemed more reliable than the stronger light source. To achieve a 100 per cent protection against failures one might of course enclose two independent lamps in the housing, but such an arrangement seemed unnecessary in the present case.

The basic problem was the design of a lamp housing which could be efficiently cooled and which could withstand the pressure of 120°C steam used for sterilization. The design is illustrated in Fig. 1.

Results and Discussion

The medium described by Vernon and Kamen[15] was only slightly altered since later modifications used in Kamen's[16] laboratory failed to improve our yields. The light intensity used in the case of the glass-flask cultivations was found to be optimum (100 lm/l.), stronger light actually depressing the growth (*cf.* Kok[24]). The illumination in the 1,000 l. fermentor was of the order of 120 lm/l. and in the 3,000 l. fermentor about 30 lm/l., figures which are not too much removed from the optimum in the glass flasks. However, the area/volume ratio was not so good in spite of the vortex aeration, a fact which may help to explain why the yields from the fermentors have been of the order of 0·5 g/l., whereas 1 g/l. could be reached in the flasks. Because many of the other factors are different, however, it is impossible to venture more than a guess in this connection. One must, for instance, remember that in the fermentors there is very little possibility of the light escaping, most of it being absorbed by the culture throughout the whole cultivation time. In the flasks, on the other hand, the utilization of the light must be lower during the first few days when the cultures are more transparent. Another factor to be considered is the possibility of nitrogen

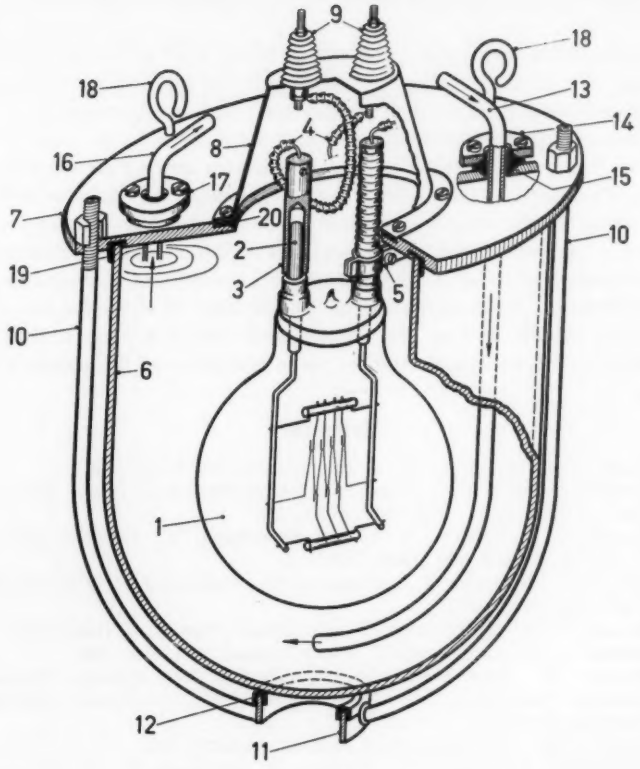

Fig. 1. Sectionalized lamp housing

The lamp (1) with its socket (2, 3) and electrical leads (4) is attached (5) inside a Pyrex glass jar (6) which is pressed against a stainless steel base plate (7) bearing a conical projection (8) with the electrical attachments (9). The jar and the base plate are held together by stainless steel rods (10) joined in a circular support (11) which is fitted with a rubber cover (12). The base plate also carries an inlet (13) for a mixture of cooling water and air, the latter providing an air cushion to surround the electrical leads and the rubber gaskets. The inlet pipe is flexibly attached to the base plate with the aid of a compression ring (14) and rubber gasket (15). The outlet pipe (16) for the cooling medium is fixed in a similar way (17). The base plate is supported by rings (18) which are attached to hooks suitably arranged around the manhole inside the fermentor. It is designed with due consideration of certain necessary rubber gaskets (19, 20).

The electrical and cooling-water attachments to the outside of the fermentors were easily arranged *via* four of the Stericonnectors attached to the top of the fermentors. Naturally the lamp was empty of cooling water when sterilized.

fixation in the fermentors, something which may well influence the yield. From the practical point of view the advantages of large-scale cultivation, of course, greatly outweigh a reduction in yield of the order just mentioned.

The harvesting in the De Laval separator is very simple but it should perhaps be pointed out that *R. rubrum*, obviously, is rather sensitive to the shock of 'shooting'. In the PX machine the rotor is opened at full speed, and the sediment is subjected to a decompression of at least 50 atm and an almost instantaneous retardation. As a result about 50 per cent of the cells are disrupted, which makes the subsequent freeze-drying and preparation of cytochrome slightly more complicated than normally.

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Aeration-agitation Studies on the Novobiocin Fermentation

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Summary. Novobiocin fermentations in 20 l. baffled fermentors were studied as a function of the size and speed of the dual four-bladed flat-blade turbines used. Power input and sulphite oxidation rate measurements were made. The courses of pH, sugar utilization, mycelial dry weight, carbon dioxide evolution and antibiotic titer were determined. Optimum antibiotic yields were achieved at a power input of 0·5 h.p./100 gal, equivalent to a sulphite oxidation rate of 110 mmoles O_2/l.h, when the impeller diameters were 29 per cent or 39 per cent of the tank diameter. A power input of 0·75 h.p./100 gal, equivalent to a sulphite oxidation rate of 160 mmoles O_2/l.h, was required for equivalent results with the impeller diameter 49 per cent of the tank diameter. Some explanations of the lack of equivalent results with the large impeller are discussed.

Introduction

Of those methods used for the estimation of oxygen transfer characteristics of fermentation vessels, the most easily applied are the sulphite oxidation rate and the power input per unit volume. The latter measurement is extremely indirect and depends to a considerable degree on maintaining geometric similitude and constant superficial air velocity between the scales of operation being compared. Its successful application in the penicillin fermentation has been reported by Wegrich and Shurter.[1]

The sulphite oxidation rate, first used by Cooper, Fernstrom and Miller[2] for study of oxygen transfer in agitated vessels, has been investigated extensively. Many workers have determined the effect of aeration-agitation in fermentors upon it[3, 4] and many have shown that it gives a good prediction of oxygen transfer rate in actual fermentations. The correlation is best with the non-viscous yeast and bacterial systems,[5–8, 34] and in some of these instances a close agreement between sulphite oxidation rate and oxygen uptake in aeration-limited fermentations was actually

observed.[9–11] Adequate scale-up of viscous, mycelial fermentations has also been achieved.[12, 13] Some of the limitations of the method have been demonstrated in publications by Wise,[14] Phillips and Johnson,[15] Pirt *et al.*[16] and Steel and Brierley.[17] The difficulty is that the chemical reaction under certain circumstances causes deviation from the diffusionally determined oxygen transfer rate. The degree of this deviation varies with agitation and may be quite minimal under normal conditions for antibiotic fermentations.[18]

Another limitation of the sulphite method is that it is measured in dilute aqueous solution, while fermentations are often highly viscous and non-Newtonian. The marked influence of suspended materials on oxygen transfer has been shown by Deindoerfer and Gaden,[19] Chain *et al.*[18] and Brierley and Steel.[20] The success of the method must be due to a maintained ratio of sulphite oxidation rate to oxygen transfer rate actually available in the fermentation under the aeration-agitation conditions being studied.

The interference of the chemical reaction may be avoided by measuring oxygen transfer rates polarographically. This method is not considered in the present report but it has been successfully applied by many workers.[18–24]

An excellent review of the subject of fermentation aeration-agitation has been published by Finn.[25]

The present paper deals with the effect of aeration-agitation in pilot equipment on the novobiocin fermentation.* This is an aerobic fermentation using a mycelial organism. The beers are pronouncedly non-Newtonian. The parameters used to measure the aeration-agitation are power input and sulphite oxidation rate. The variations in tank geometry, agitation rate and air flow are well within the normal range employed for antibiotic fermentations, yet it has been possible to demonstrate clearly some limitations of these parameters in predicting fermentation behaviour.

Equipment and Procedures

Fermentor

The fermentor, seed vessel, and auxiliary equipment used in these experiments have been described previously.[26] The geo-

* Smith[33] has recently completed some shaken flask studies on aeration effects in the novobiocin fermentation. He studied the interaction of mycelium concentration and aeration with respect to its influence on novobiocin titers.

metry of the fermentor agitation system was considerably modified, however. Fig. 1 illustrates the significant dimensions: two four-bladed flat-blade turbine impellers and four symmetrically spaced baffles were employed. Three sizes of impeller were available, their over-all diameters being 29 per cent, 39 per cent and 49

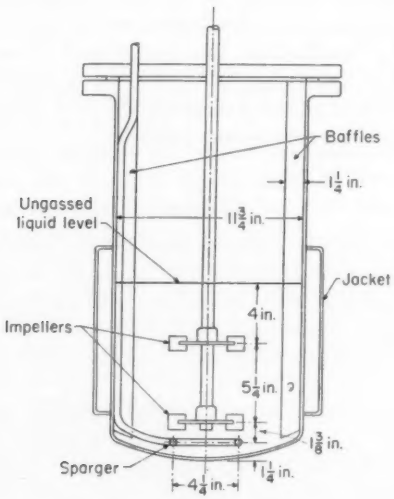

Fig. 1. Fermentor interior

Small impeller: over-all diameter 3·44 in.; blades 0·72 × 0·86 in.
Standard impeller: over-all diameter 4·63 in.; blades 0·94 × 1·19 in.
Large impeller: over-all diameter 5·81 in.; blades 1·19 × 1·44 in.

per cent of the inside tank diameter. The blade dimensions were proportional to the diameter in each case.

All runs were made with a liquid volume of 20 l. in the fermentor after inoculation. The air-flow rate was held constant at 2·2 SCFM (standard ft^3/min humidified air measured at 14·7 lb/in^2 and 70°F) and the pressure in the tank maintained at 5 lb/in^2 gauge for all runs.

Fermentation

Strain 3R of the novobiocin-producing culture *Streptomyces niveus* was used in a medium containing (after sterilization and inoculation) 40 g/l. of Brown–Forman distillers' solubles and 40 g/l. of Cerelose (Corn Products Refining Co., glucose monohydrate). Sufficient sodium hydroxide solution was added to raise the pH before sterilization to 8·0. Vegetative inoculum, 5 per cent by volume, grown for 3 days on the same medium (but with the pH *after* sterilization readjusted to 8·0) was used. The fermentations were continued for 115 h. Lard oil containing 1 per cent octadecanol was added automatically as a defoaming agent; about 50 ml was the average consumption.

Analyses

The antibiotic titer was determined by the ultraviolet method described elsewhere.[27] Results are expressed as μg/g of whole beer (weighed sample). All components of the novobiocin complex are included. The microbiological assay procedure[27] was used as a check and ordinarily gave slightly lower, more variable, results.

The sugar determination, a modified Somogyi method,[28] was run on unhydrolyzed filtered beer against a glucose standard.

For mycelial dry weight measurement, an aliquot of filtered beer was centrifuged under reproducible conditions in a tared, calibrated tube. The percentage sediment was measured and the solids washed two or three times and dried *in vacuo* at 60°C to constant weight. Since, during the fermentation, mycelium and medium solids both contribute significantly to the total dry weight, an equation involving both percentage sediment and dry weight was used to calculate the approximate *mycelial* dry weight:

$$\text{mycelial dry wt.} = \frac{S - (S/D)_0 D}{(S/D)_{\max} - (S/D)_0}$$

where S = sediment, ml/l., D = total dry weight, g/l. The subscript '0' refers to the initial pre-inoculation conditions and 'max' refers to the maximum achieved during the fermentation.

The method's validity is based on two assumptions:

(1) Dry weight and sediment due to insoluble material from the medium are negligible late in the fermentation. Visual and microscopic observation indicated that this was true.

(2) The ratio of dry weight to percentage sediment remains constant for the mycelium and the medium solids independently through the run. This assumption could not easily be verified.

This method is of dubious accuracy but no more satisfactory procedure for the determination of mycelial weight in the presence of medium solids is available.

The method for sulphite oxidation rate determination was modified somewhat, for the sake of convenience, from that used by Cooper, Fernstrom and Miller.[2] In our procedure the fermentor was filled to the 20 l. level with a solution of sodium sulphite of proper concentration (1·0 N was usual), to which was then added a concentrated solution of cupric sulphate to a level of 0·005 M. After air and agitation were started, a sample was withdrawn into a flask. A measured aliquot of this sample (usually 5 ml) was pipetted into a test tube containing a small piece of dry ice, which blanketed the sample with carbon dioxide to terminate further oxidation. The sulphite solution was then titrated to the starch endpoint with an iodine solution of appropriate normality. The sampling was repeated at timed intervals. A plot of titration against time was prepared, and the slope of a straight line determined by four or five points (expressed as mmoles O_2/l.h) was taken as the sulphite oxidation rate.

Instrumentation

The torsion dynamometer used for power measurements and the recording infrared analyzer used for determination of carbon dioxide were described in an earlier publication.[26] A modified Stormer viscometer was used for rheological measurements. This measured the rate of turn of a two-bladed fork-shaped paddle in the fluid in question under the application of different amounts of torque.

Results

Physical Measurements

The effect of agitator speed and size on the power input to the water in the fermentor is shown in Fig. 2. The slope of the lines on this log-log plot was drawn equal to 3·0 ($P = KN^3$). This relationship is expected from the work of previous investigators.[29]

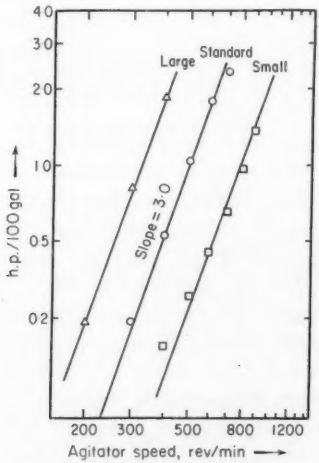

Fig. 2. Power input to water in the 20 l. fermentor for three impeller sizes

Experimental points fall, within error, on the lines. A cross plot shows that power input varies, as expected, with agitator diameter to the 5th power ($P = K'D^5$).

Power inputs to actual fermentation beers were also measured in several instances. In spite of the much higher viscosities of these beers the results were equivalent to those with water within the accuracy of the measurement ($\pm$ 10 per cent). Furthermore, no significant variation in power during the course of a fermentation was noted. Published power correlations predict no effect of viscosity under conditions of high turbulence.[29] The rheological behaviour of beer samples taken from a typical fermentation is shown in Fig. 3: a shear diagram obtained from measurements in

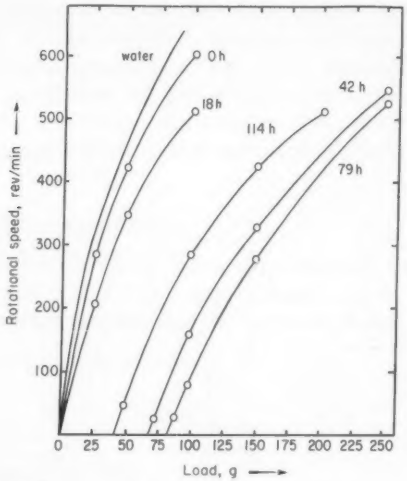

Fig. 3. Shear diagram of typical novobiocin beers

Age of fermentation at time of sampling is given in hours.

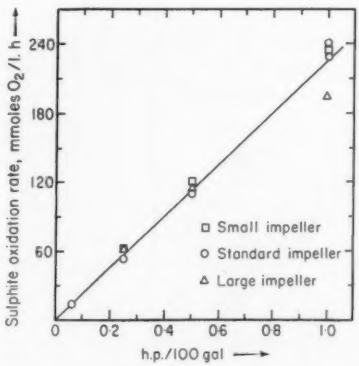

Fig. 4. Effect of power input and impeller size on sulphite oxidation rate

the Stormer viscometer. The differences in apparent viscosity from one time to another during the fermentation can be seen to be due more to the changes in yield point than to the changes in the slope of the lines. The curvature of the lines is real, but exaggerated by frictional resistances in the viscometer, as is evidenced by the curvature of the line for water.

Fig. 4 shows the sulphite oxidation rates obtained as a function of the h.p. input using the three agitator sizes. Except for

one point with the large impellers at 1·0 h.p./100 gal, the rates are directly proportional to power input regardless of agitator size. Thus, sulphite number and power input are equivalent parameters for correlation of fermentation behaviour within the range where antibiotic yield effects occur. The one point of deviation falls outside this range.

Fermentation Behaviour

A series of novobiocin fermentations was run over a range of power inputs from 0·1–1·0 h.p./100 gal for each of the three agitator sizes. Measurements were made of pH, sugar, mycelial

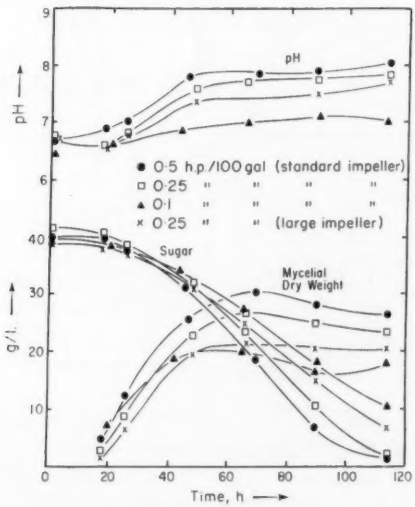

Fig. 5. Changes in pH, mycelial dry weight, and sugar during the fermentation, as a function of agitation

dry weight, novobiocin titer and carbon dioxide evolution rate. The results of typical runs are plotted in Figs. 5 and 6, giving concentration or rate against fermentation time. A key index, the novobiocin titer at 115 h, is plotted against the agitation conditions, power input and impeller size, in Fig. 7.

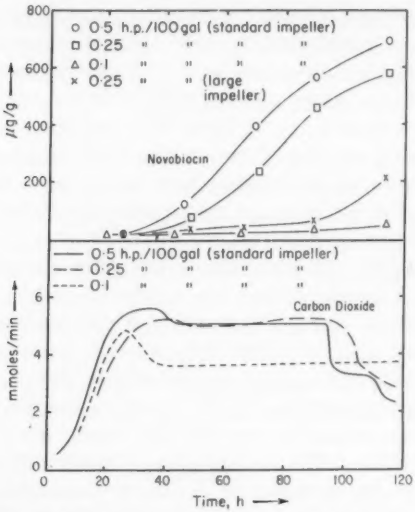

Fig. 6. Changes in novobiocin titer and carbon dioxide evolution rate during the fermentation, as a function of agitation

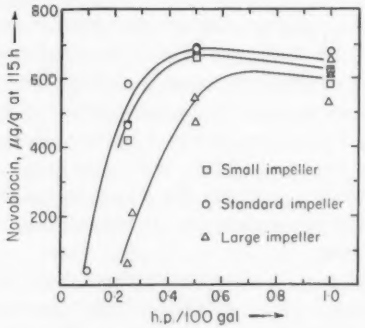

Fig. 7. Effect of power input and impeller size on harvest yield of the novobiocin fermentation

For the small and standard impellers the effect of power input (or of sulphite oxidation rate) on novobiocin titer is similar. Antibiotic yield increases from near zero at 0·1 h.p./100 gal to an

optimum at 0·5 h.p./100 gal. Examination of Figs. 5 and 6 shows that antibiotic production is the most sensitive to aeration-agitation of those factors measured. Limiting conditions cause not so much a change in rate, as a delay in the beginning of production. This results in a striking effect on the 115 h yield level. Less pronounced are the effects on the other factors; insufficient agitation-aeration is manifested by a lower pH level during the antibiotic production phase, a less rapid utilization of sugar, and a smaller amount of mycelial dry weight. Effects on carbon dioxide evolution rate are even less pronounced. No reduction is noted at 0·25 h.p./100 gal even though the antibiotic yield is about 70 per cent of optimum. At 0·1 h.p./100 gal carbon dioxide evolution rate is reduced only by about 30 per cent, but the yield is near zero. Even this effect can be noted only in the period after antibiotic production begins. In the most active period of growth carbon dioxide evolution reaches the same level regardless of aeration-agitation. The maintenance of carbon dioxide evolution may be due to the presence of anaerobic dissimilation pathways. One might speculate that the shift from aerobic to anaerobic metabolism has a pronounced effect on the elaboration of the rate-limiting enzyme for novobiocin synthesis.

While 0·5 h.p./100 gal is sufficient power for optimum yield with the small and standard impellers, the yield at this power level is only 75 per cent of optimum when the large impellers are used. This difference is statistically significant at the 5 per cent level. The reduced yield is even more noticeable at 0·25 h.p./100 gal. Interpolation on the curves in Fig. 7 indicate that 0·75 h.p./100 gal is required for optimum yield with the large impellers. Fermentations at 1·0 h.p./100 gal show that when this power level is reached the antibiotic yields are approximately equivalent for all three impeller sizes.

While the evidence is only presumptive, it would appear that the reason for reduced yield with the large impellers at less than 0·75 h.p./100 gal is inadequate aeration. The curves in Figs. 5 and 6 indicate the same effects as occur with the smaller impellers under insufficient aeration-agitation, i.e. a lower pH course, less mycelial dry weight, reduced rate of sugar consumption and a delay in time of antibiotic production rather than a decrease in rate. If this assumption is correct, it must be concluded that

neither power input per unit volume nor sulphite oxidation rate, the two factors being directly interconvertible, according to Fig. 4, in the region below 0·5 h.p./100 gal, is a satisfactory index of aeration-agitation with mycelial fermentations under all conditions of agitator geometry.

There is an indication, though not statistically significant at the 5 per cent level, that yields of novobiocin are reduced under conditions of excessive agitation. Dion *et al.*[30] have shown such a phenomenon to occur in the penicillin fermentation. They attribute the effect to mechanical damage to the mycelium, but observe similar damage at lower agitation levels when air is replaced by oxygen. Excessive agitation results in lower yields in a kojic acid fermentation according to Camposano *et al.*,[31] and Steel[32] has shown reduced yields with high agitation in an actinomycete fermentation under conditions of constant oxygen supply.

Discussion

There may be several reasons for the observed lack of correlation between sulphite oxidation rate or power input on the one hand and antibiotic yield on the other. Basically, most of these resolve to the simple proposition that these parameters are not always proportional to the oxygen transfer rate actually available in a non-Newtonian fermentation beer. Just where the deviation occurs is open to speculation. Some of the alternatives are:

(1) Sulphite oxidation rate is not a true, or even proportional, measure of the maximum oxygen transfer rate, even in a dilute aqueous system as measured, for example, by polarographic procedures. Wise,[21] and more recently Phillips and Johnson,[15] have shown how the chemical reaction may cause higher oxygen transfer than occurs by purely diffusional means. The degree of this discrepancy is dependent on the agitation characteristics and may not be appreciable under the conditions employed in most antibiotic fermentations. It is, however, possible that the large impeller gives a higher ratio of sulphite-measured oxygen transfer to purely diffusional oxygen transfer than the two smaller sizes.

(2) Oxygen transfer rate in a dilute aqueous system is not a true, or even proportional, measure of the oxygen transfer rate in a viscous

non-Newtonian fermentation beer. Several workers have shown how added mycelium or other fibrous materials reduces polarographically measured oxygen transfer. For this to explain the present observations, however, requires that the effect of mycelium is *greater* proportionally with the large impeller than with the two smaller impellers. Such might well be the case since the flow component of the power consumption (proportional to ND^3) is greater and the turbulence component (proportional to N^2D^2) is less with the large impeller. This reduced turbulence may have a more pronounced effect on oxygen transfer in the viscous than the non-viscous system.

(3) Non-homogeneity of oxygen supply may exist in the fermentor and its degree may be influenced by the impeller size. It is likely that the larger impeller with its higher flow component would cause a more uniform distribution of oxygen supply throughout the vessel. With the smaller impellers regions of local high turbulence and oxygen transfer could be visualized even though the over-all transfer would remain unchanged. This situation might cause the observed effect on antibiotic yield.

Which, if any, of these three alternatives is operating in the present case is not demonstrated. Perhaps it is a combination of them all. Effort to elucidate the phenomenon is continuing in these laboratories. The studies reported here serve to illustrate that, while sulphite oxidation rate and power input are useful parameters for estimating oxygen transfer rate in fermentations, they are applicable only within certain limits. This work partially indicates these limits.

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Distribution of Bacterial Cells within Fibrous Air Sterilization Filters

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Summary. Probability theory has been applied to the analysis of bacterial distribution within a fibrous air sterilization filter. For a long filter layer, to which the so-called 'log-penetration' law is no longer applicable, the probability theory is considered to be effective in interpreting the distribution data.

The effects of the diameter, volume fraction and moisture content of the glass-fibre on the longitudinal distribution of bacterial cells, were studied. The distribution was estimated beyond the experimental data points. This estimation will be of use for predicting the 'life' of an air sterilization filter.

Introduction

The importance of air sterilizing filters in the fermentation industry cannot be exaggerated; but the general lack of information on the sterilizing capacity of such filters has made their rational design impossible.

To remedy these conditions, it is necessary, first, to study the distribution of bacterial cells within filters. Recently several investigators have published studies on the distribution of bacterial cells within air filters, made of glass or of porous materials.[3,4] In all these publications, the 'log-penetration' relation has been employed. This relationship is, however, likely to be observed only in rather thin filters, less than 1 cm for example. In practice, the use of such a thin filter is limited.

Therefore, the necessity of studying the distribution in thicker layers of filter arises. In this paper, endeavours were focused on analysis of the distribution, so that an estimate of the bacterial

distribution in air filters could be made practically feasible. Improved information on distribution will contribute not only to the fermentation industry, but also to aerosol filtration problems in other processes.

Theoretical considerations

For the purpose of analysing the 'number' concentration gradient of bacterial cells within the filter, it is assumed that the filter is composed of a large number of grids of glass-fibre. In passing through these grids, the bacterial cells have a probability p of collision with each set of grids. The probability p is considered to depend mainly on the dimensions of the bacterial cell and the mesh of the grid, and, in particular, on the fluid flow pattern of the bacteria-bearing air through the filter.

Analyses of the probability, from the fluid dynamics viewpoint, have been reported by several workers.[2,3] In these analyses, the re-entrainment of bacterial cells or small particles into the aerosol stream is not specifically taken into account.

In this paper, it is assumed that, within a certain length of air filter, even those bacterial cells which make, on an average, as many as m collisions with the grids have a possibility of escape out of the filter. (The practical meaning of m will be referred to later.)

The number of bacterial cells retained within a filter of length L, is then given by

$$\nu = \nu_0\left\{1 - \sum_{m=0}^{m=m} {}_{\xi L}C_m \cdot p^m \cdot (1-p)^{\xi L - m}\right\} \qquad (1)$$

where ξ is the number of grids per unit length of filter, ν_0 is the total number of bacterial cells blown into the filter and ν is the number of bacterial cells captured by the filter.

In equation (1), when $m = 0$,

$$\nu = \nu_0\{1-(1-p)^{\xi L}\}$$

and

$$\begin{aligned}\ln(1-\nu/\nu_0) &= \xi L \ln(1-p) \\ &= \xi L(-p-p^2/2!-p^3/3!-\ldots)\end{aligned}$$

If, in the above derivation, p is small enough for only the first term to be taken, then

$$\nu \simeq \nu_0(1-\mathrm{e}^{-\xi Lp}) \tag{1'}$$

and

$$\frac{\mathrm{d}\nu}{\mathrm{d}L} \simeq \nu_0 \xi p \mathrm{e}^{-\xi Lp} \tag{2}$$

Equations (1') and (2) correspond to the 'log-penetration' law.

In equation (1), when $m = 1$, $\xi L - 1 \simeq \xi L$ and $p \ll 1$,

$$\frac{\mathrm{d}\nu}{\mathrm{d}L} \simeq \nu_0 \xi^2 p^2 \mathrm{e}^{-\xi Lp}(L/1!) \tag{3}$$

In the case of fibres several μ in diameter, ξ is assumed to be several hundred per cm of filter.

For $m = 2$ and 3 respectively, taking only the highest power of ξLp and neglecting all other terms, equations (4) and (5) are derived.

$$\frac{\mathrm{d}\nu}{\mathrm{d}L} \simeq \nu_0 \xi^3 p^3 \mathrm{e}^{-\xi Lp}(L^2/2!) \tag{4}$$

$$\frac{\mathrm{d}\nu}{\mathrm{d}L} \simeq \nu_0 \xi^4 p^4 \mathrm{e}^{-\xi Lp}(L^3/3!) \tag{5}$$

The error in deriving equations (2)–(5) is considered to be less than one per cent.

In general when $m = m$

$$\begin{aligned} \frac{\mathrm{d}\nu}{\mathrm{d}L} &= \nu_0' \xi p \mathrm{e}^{-\xi Lp}[(\xi Lp)^m/m!] \\ &= \nu_0' k \mathrm{e}^{-kL}[(kL)^m/m!] \end{aligned} \tag{6}$$

where $k = \xi p$

$$\nu_0' \int_0^\infty k \mathrm{e}^{-kL}[(kL)^m/m!]\mathrm{d}L \equiv \nu_0$$

The reason for introducing the term ν_0' is to compensate for errors resulting from the approximations made in equations (2)–(5).

Experimental Apparatus and Procedure

The experimental apparatus and procedure are similar to those reported in a previous paper.[1] The suspension of ^{32}P-labelled

Serratia marcescens bacteria in distilled water was atomized by air from a compressor. The relative humidity of the bacteria-bearing air was adjusted to about 70 per cent and the bacterial concentration used was of the order of 10^8 cells/m^3 of air. The cells had a diameter of about 1 μ.

The bacterial aerosol was filtered using a filter packed with five or six successive pads of glass-fibre, each filter pad being about 10 mm in thickness. The filter vessel, 100 mm in diameter and made of brass and acryl resin, was also used in this series of experiments. The nominal velocity of air through the filter was 5 cm/sec throughout.

The radiation emitted from the bacterial cells retained in a certain volume (about 1·0 cm diam. × 1·0 cm) of each pad, after a certain period of operation, was also determined by the same procedure as previously described,[1] using a Geiger-Müller counter. The surface-mean of the radiation intensities, which were measured ten times along arbitrarily chosen radial directions in each pad, was assumed to represent the numerical concentration of cells in the pad.

In this report, the effects of a number of variables on the distribution were studied. These variables, together with their experimental ranges, are

(a) fibre diameter (d_f = 8–19 μ) (moisture content w = 0 per cent).
(b) volume fraction of the fibre (α = 2·5–3·8 per cent).
(c) moisture content of the filter (w = 0–84 per cent).

For ease of handling, the glass fibres of each diameter were made into thin sheets, 100 mm in diameter. Each filter pad, of about 10 mm thickness, was comprised of a large number of these sheets and could be tightly fitted in the filter vessel.

The volume fraction of the fibre was varied by changing the number of sheets which make up each pad. Due to the elastic nature of the pad, the range of α in these experiments was rather narrow. However, in relation to practice in industrial filtration, the experimental range of α is not too limited.

The principal aim of studying the effect of the moisture content of the filter is to know the necessary measure of drying required just before the operation of the filter. In practice, steam is

usually blown through the filter to sterilize the fibre, and the fibre then de-humidified with hot air. It is generally advocated that the fibre is dried completely, because residual moisture is said to reduce filter efficiency. To simulate the experimental conditions, each pad was dipped into water and then de-humidified in an electric oven until the experimental value of the moisture content was reached. These pads were packed in the filter vessel to determine the distribution, in the same way as those which were studied without such processing. Since the moisture content changes during the aerosol filtration, the arithmetic mean of the content, obtained by weighing each pad before and after each experiment, was chosen as the representative value.

Experimental Results and Discussion

Assuming that the number of bacterial cells within each filter pad is proportional to the radiation intensity n emitted from the pad, equation (6) can be modified as follows:

$$\frac{\mathrm{d}n}{\mathrm{d}L} = n_0' k \mathrm{e}^{-kL}[(kL)^m/m!] \qquad (6')$$

where $n = \beta\nu$ and $n_0' = \beta\nu_0'$.

An example of a histogram of the longitudinal distribution for $d_f = 8\ \mu$, $\alpha = 3{\cdot}3$ per cent, $w = 0$ per cent and $\theta = 3$ h is shown in Fig. 1, the ordinate and abscissa being the gradient of bacterial concentration and the filter depth, respectively.

One would expect the data points A, B, C, D and E to give a continuous gradient curve, but even so, it is difficult to know, in advance, to what portion of the continuous curve the 'log-penetration' law should be applied.

Conversely, postulating the log-penetration phenomenon, and that the condition of $m = 0$ is predominant in a rather thin layer at the filter inlet, it is assumed that the condition is satisfied in the region represented by drawing a straight line through the points A and B. These points correspond to the values obtained in the first and second filter pads along the direction of air flow. The slope of this straight line determines k, and its intersection with the ordinate gives $n_0'k$. Then using each value of $(\mathrm{d}n/\mathrm{d}L)$ from C to E, together with the pre-determined values

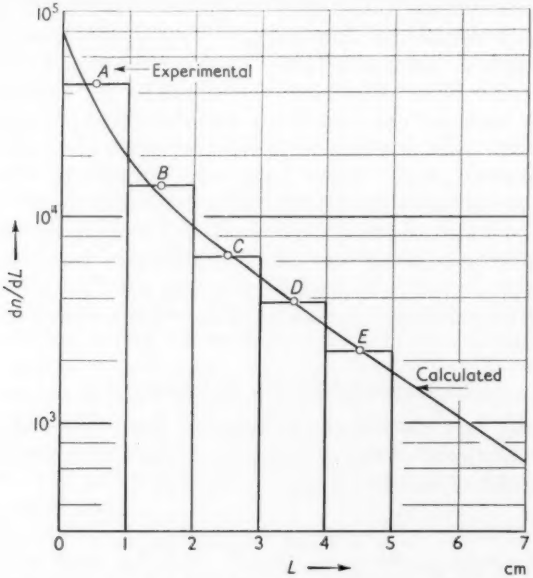

Fig. 1. An example of a histogram of the bacterial distribution
$d_f = 8\ \mu$, $w = 0$ per cent, $\alpha = 3{\cdot}3$ per cent and $\theta = 3$ h

of n_0' and k, m is calculated by satisfying equation (6′). These estimated values of m are plotted against the corresponding filter length L in Fig. 2. The relation between L and m is linear.

Assuming that the extrapolation of the linear relationship holds beyond the experimental range of L, $(\mathrm{d}n/\mathrm{d}L)$ can be calculated using equation (6′). The result of this calculation is included in Fig. 1. The discrepancy near the data points A and B is probably due to the original approximation of drawing a straight line through A and B.

If the region over which it is assumed $m = 0$ is varied, it is found that a linear relationship, as shown in Fig. 2, is obtained in each case, although the numerical values of m are different. Briefly, m indicates the deviation of the gradient curve from each straight line representing the condition $m = 0$. Therefore, m depends on how this straight line is drawn. Theoretically, the

coincidence between experimental and calculated data in Fig. 1 should be closest when the most appropriate choice for the region in which $m = 0$ is made.

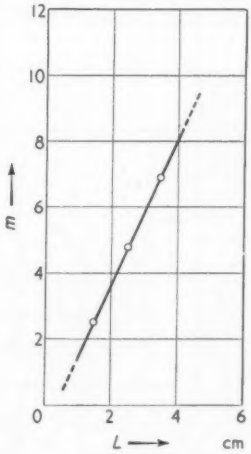

Fig. 2. An example of the relationship between m and L

The data for m are empirical. The maximum deviation resulted from the error in the longitudinal distribution data for the bacterial cells, of about ± 3 per cent, and is estimated to be of the order of $m \pm 0{\cdot}05$. Since the gradient curve (Fig. 1) is shifted parallel to itself with variation in the operation time θ, m should be independent of θ.

The effects of fibre diameter, volume fraction and moisture content on the longitudinal distribution of bacterial cells are summarized in Figs. 3, 4, and 5. The ordinates of the figures represent $(\mathrm{d}n/\mathrm{d}L)$ and the abscissae the depth of filter L. For easy comparison of data, $(\mathrm{d}n/\mathrm{d}L)$ for the first pad is arbitrarily chosen as unity. The relations between m and L are also shown in the figures, the procedure for evaluating m being exactly the same as that described for Fig. 1.

Fig. 3 shows clearly the marked effect of fibre diameter on the gradient, and indicates that the glass-fibre of diameter 19 μ is the least effective in the filtration of bacterial cells. Fig. 4 shows that a slight change in the volume fraction α exerts a conspicuous effect on the distribution of bacterial cells within the

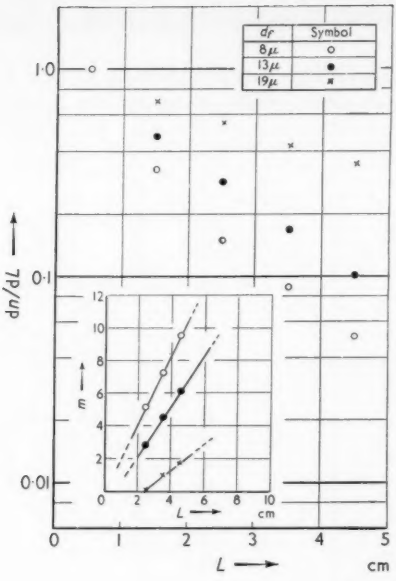

Fig. 3. Effect of glass-fibre diameter on the bacterial distribution

$\alpha = 3{\cdot}3$ per cent, $w = 0$ per cent

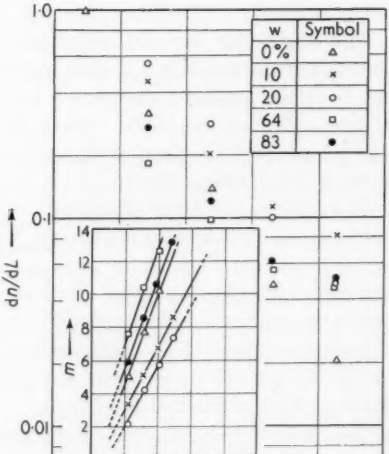

Fig. 4. Effect of volume fraction of glass-fibre on the bacterial distribution

$d_f = 8\ \mu$, $w = 0$ per cent

filter. The experimental result that the larger fraction seems more effective in filtration agrees with theory, because the number of grids of glass-fibre per unit length of filter, through which the bacteria-bearing air passes, increases with increase of α.

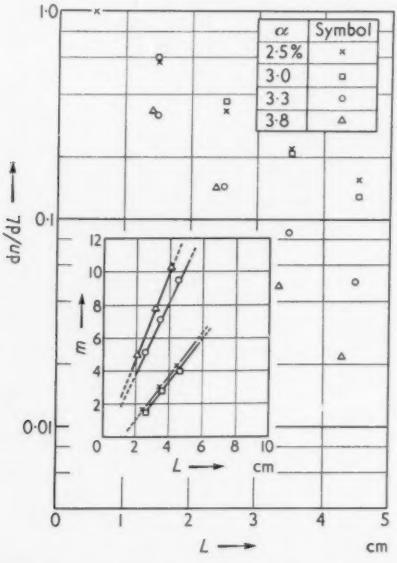

Fig. 5. Effect of moisture content of filter on the bacterial distribution

$d_f = 8\ \mu$, $\alpha = 3{\cdot}8$ per cent

It was believed that the moisture content had an adverse effect on filtration. The quantitative relationship between the moisture content w and the deterioration in filtration efficiency is not always apparent, however (Fig. 5). This may be partly caused by the dislocation of glass fibres as de-humidification proceeds after dipping the fibre in water.

Because of the experimental result that the relationship between (dn/dL) and L is not linear when plotted on semi-logarithmic paper, the collection efficiency advocated by Langmuir *et al.*[2,3] varies along the filter length.

Since the effects of various factors on the collection efficiency can be noted intuitively from these figures, correlation between the efficiency and each experimental condition is not attempted in this paper.

Nomenclature

d_f Diameter of glass-fibre in μ

k ξp

L Length of filter, cm

m Number of times which bacterial cells, once they collide with a grid, are re-entrained into the bacteria-bearing air flow

n Intensity of β-rays emitted from the bacterial cells, counts/5 min

p Probability that the bacterial cells collide with the grid structure of the glass-fibre

w Moisture content, per cent

α Volume fraction of glass-fibre

β Proportionality constant

ν Number of bacterial cells captured by the filter

ν_0 Total number of bacterial cells blown into the filter during the operational period θ

ν_0' $\nu_0 \Big/ \int_0^\infty k e^{-kL} \dfrac{(kL)^m}{m!} \, dL$

ξ Number of grids of glass-fibre per cm of filter

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